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Review

Advances in the Application of CRISPR/Cas Systems in Molecular Diagnostics

Haozhe Ren [1,2,3], Shiyuan Luo [4], Yiqing Yu [5], Pengfei Chen [6,7,*] and Ke Wang [1,*,†]

[1] Department of Pharmaceutical Analysis, School of Pharmacy, The Fourth Military Medical University, Xi'an 710032, China; renhzh2023@lzu.edu.cn (H.R.)
[2] School of Stomatology, Lanzhou University, Lanzhou 730000, China
[3] Department of Preventive Dentistry, School of Stomatology, The Fourth Military Medical University, Xi'an 710032, China
[4] The Twenty-Second Squadron of the Sixth Regiment, School of Basic Medicine, The Fourth Military Medical University, Xi'an 710032, China; luoshiyuan@fmmu.edu.cn (S.L.)
[5] The Ninth Squadron of the Third Regiment, School of Basic Medicine, The Fourth Military Medical University, Xi'an 710032, China; yuyiqing@fmmu.edu.cn (Y.Y.)
[6] Department of Medical Oncology, The Second Affiliated Hospital of Xi'an Jiaotong University, Xi'an 710004, China
[7] Institute of Medical Engineering, School of Basic Medical Sciences, Xi'an Jiaotong University, Xi'an 710061, China

* Corresponding author. E-mail: chenpengfei@xjtu.edu.cn (P.C); wangke123@fmmu.edu.cn (K.W.)

† This author is the primary corresponding author.

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ABSTRACT: Rapid advances in CRISPR/Cas systems and the growing global demand for rapid, accurate diagnostics underscore the necessity of reviewing how these technologies are transforming molecular testing. Conventional diagnostic approaches are frequently constrained by prolonged turnaround times, complex instrumentation, and limited analytical sensitivity, and these limitations were starkly highlighted during the COVID-19 pandemic. In this context, we present a comprehensive and timely overview of CRISPR/Cas-based molecular diagnostics. We begin by summarizing the classification and molecular mechanisms of CRISPR/Cas types I–VI, followed by a detailed discussion of innovative detection strategies such as SHERLOCK, DETECTR, and amplification-free platforms that significantly enhance analytical sensitivity and specificity. We further explore clinical applications across infectious disease surveillance, antimicrobial resistance profiling, early cancer detection, genetic variant identification, and the emerging detection of non-nucleic acid biomarkers. Finally, we discuss future perspectives, including the development of miniaturized, high-throughput, and AI-assisted diagnostic platforms, their integration with microfluidics and portable readout systems for point-of-care applications, and highlight critical challenges such as standardization, automation, and cost-effectiveness that must be addressed to facilitate clinical translation.

Keywords: CRISPR/Cas system; Molecular diagnostics; Point-of-care testing (POCT); Clinical translational application; Artificial intelligence-assisted molecular diagnosis

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1. Introduction

1.1. Mechanisms and Taxonomy of CRISPR/Cas Systems

CRISPR/Cas systems mediate an adaptive immune response in bacteria and archaea by the programmable, sequence-specific recognition and endonucleolytic cleavage of foreign nucleic acids. Phylogenomic analyses led Makarova et al. [1] to propose a widely adopted classification that divides these systems into Class I (multi-subunit effector complexes) and Class II (single-protein effectors). Owing to distinct effector-architectures, each class is further subdivided into six major types (I–VI), whose key molecular features are summarized in Table 1.

Table 1. The relation of CRISPR/Cas system effector proteins.

Cas Protein	Class	Type	Target Nucleic Acid	Trans-Cleavage Activity/Substrate	PAM/PFS Sequence	References
Cas9	Class II systems	Type II	dsDNA	No	3′ GC-rich PAM	[2,3]
Cas7–11 (Multi-subunit effector)	Class I systems	Type III	ssRNA	No	---	[4–6]
Cas12a (Cpf1)	Class II systems	Type V	dsDNA/ssDNA	Yes/ssDNA	5′ AT-rich PAM	[7]
Cas12b	Class II systems	Type V	dsDNA/ssDNA	Yes/ssDNA	5′ AT-rich PAM	[8]
Cas12f (Cas14)	Class II systems	Type V	dsDNA/ssDNA	Yes/ssDNA	5′ T-rich PAM	[9–13]
CasΦ (Cas12j2)	Class II systems	Type V	dsDNA/ssDNA	Yes/ssDNA	5′ TBN	[14]
Cas13	Class II systems	Type VI	ssRNA	Yes/ssRNA	3′ non-G-PFS	[15]
Cas3 protein (Multi-subunit effector)	Class I systems	Type I	dsDNA	Yes/ssDNA	AAG	[16]

Note: Cas9 exhibits trans-cleavage activity, but this activity strictly requires a tracrRNA–crRNA duplex and cannot be elicited by a single-guide RNA (sgRNA) alone [17].

Class I effectors are multi-subunit ribonucleoprotein complexes represented by types I, III, and IV. The signature Cascade complex assembles Cas5, Cas6, Cas7, Cas8, and Cas11 to process pre-crRNA, recognize target sequences, and catalyze nucleic-acid cleavage. Pronounced differences in subunit composition between subtypes substantially enhance system adaptability [1]. However, the inherent macromolecular complexity, large molecular mass, and intricate regulation have largely excluded these systems from the diagnostic arena. Cas7–11 is a streamlined chimeric effector comprising four Cas7 domains fused to a single Cas11 domain. It overcomes these constraints by executing crRNA-guided RNA cleavage as an autonomous entity [6]. This self-contained architecture obviates auxiliary factors and collapses multistep workflows into a single reaction. Rational reprogramming of its RNA-recognition surface or seamless integration with microfluidic multiplexers now positions Cas7–11 as a complementary platform to Class 2 effectors, enabling simultaneous pathogen screening and tumor-marker quantification, thereby broadening the CRISPR diagnostic ecosystem.

Class II immunity is mediated by single, monolithic effector proteins that have transformed genome engineering. Encompassing types II, V, and VI, this class deploys four canonical enzymes, Cas9, Cas12, Cas13, and Cas14, whose distinct substrate preferences span dsDNA, ssDNA, and RNA, thereby enabling versatile, multiplexed applications in gene editing and molecular diagnostics (Figure 1). The archetypal type II module centers on Cas9, which interrogates dsDNA via a crRNA–tracrRNA duplex (or single-guide RNA surrogate) and obligately engages a protospacer-adjacent motif (PAM). Conformational activation positions the RuvC and HNH nuclease domains for synchronized incision, yielding a staggered double-strand break with single-base precision [18,19]. Within the type II machinery, the complementary DNA strands are simultaneously docked, with the target strand engaging the HNH pocket and the non-target strand occupying the RuvC groove. Product entrapment within these catalytic clefts renders Cas9

catalytically quiescent, precluding subsequent turnovers. The type V repertoire employs the monomeric Cas12 architecture. In the paradigm V-A effector Cas12a (formerly Cpf1), a solitary RuvC domain is directed by a single crRNA to protospacer-adjacent motif (PAM)-flanked dsDNA. Upon complete complementarity, Cas12a initiates a sequential cis-cleavage cascade process in which the non-target strand is cleaved first, followed by incision of the target strand, thereby producing a precise double-strand break while maintaining enzymatic efficiency. After cis-cleavage of target dsDNA, Cas12a undergoes a conformational switch that unleashes indiscriminate, trans-acting DNase activity against non-target ssDNA. This trans-cleavage activity is converted into a fluorescence burst via rapid turnover of quenched ssDNA reporters, affording an ultrasensitive signal amplification module for diagnostic assays. Notably, crRNA-complementary single-stranded DNA can also activate Cas12a in a PAM-independent manner, eliciting efficient endonucleolytic cleavage. Cas12b exhibits exceptional thermostability within the type V-B clade, extending its utility to field-deployable point-of-care testing [8]. Cas12f (type V-F, previously Cas14) is the most compact CRISPR nuclease yet identified, spanning only 400–700 amino acids. Guided by a single crRNA, it binds cognate ssDNA and catalyzes indiscriminate trans-cleavage of bystander ssDNA, providing a minimal, high-fidelity platform for attomolar-level nucleic-acid detection without pre-amplification [9]. Compared with Cas9, Cas12 exhibits slower kinetics but substantially higher fidelity, reducing off-target activity to near-background levels. In contrast, type VI systems operate as RNA-specific ribonucleases. Upon crRNA-guided binding of a cognate transcript, Cas13a (type VI-A) undergoes allosteric activation that aligns its dual HEPN nuclease domains and triggers trans-RNase activity against proximal ssRNA. By encoding orthogonal crRNAs for each target and pairing them with spectrally distinct fluorescent reporters, single-reaction multiplexed detection of diverse analytes is achieved [15,20–22]. The interchangeable modularity and multiplexing capability inherent to Class 2 systems provide the foundation for advanced CRISPR-based diagnostics that achieve attomolar sensitivity while remaining directly deployable in both clinical laboratories and point-of-care settings.

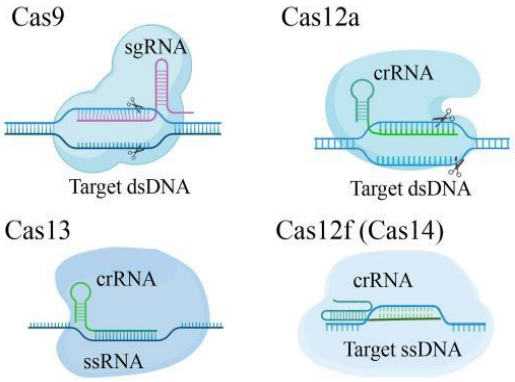


Figure 1. Schematic diagram and principles of CRISPR/Cas systems for biosensing and bioimaging.

1.2. Innovation and Optimization of CRISPR-Based Diagnostic Platforms

The emergence of CRISPR in nucleic-acid diagnostics reflects the combination of molecular precision and structural versatility. Through canonical Watson–Crick base pairing, a single rationally designed crRNA provides single-nucleotide specificity, enabling unambiguous discrimination of pathogen sequences or host mutations. Cas12 and Cas13 exploit orthogonal collateral pathways. dsDNA-bound Cas12 elicits trans-DNase activity that indiscriminately degrades ssDNA reporters, while RNA-bound Cas13 activates trans-RNase activity that non-specifically cleaves proximal ssRNA. Both cascades convert the primary recognition event into an amplified signal via rapid reporter turnover. Leveraging this intrinsic enzymatic amplification, the crRNA scaffold can be iteratively reprogrammed to detect DNA, RNA, or non-nucleic-

acid analytes while remaining compatible with fluorescence, electrochemical, or colorimetric readouts. These features establish a modular engineering framework for portable, high-throughput diagnostic devices.

1.2.1. CRISPR-Based Diagnostic Technologies Employing Nucleic Acid Pre-Amplification

CRISPR/Cas systems achieve single-base specificity. However, their intrinsic detection limit is typically in the pM–fM range and remains insufficient for clinical trace analytes such as attomolar viral nucleic acids. To address this limitation, researchers have developed pre-amplification/CRISPR synergistic strategies that combine nucleic acid amplification with CRISPR trans-cleavage activity, achieving a 3–6 orders of magnitude improvement in analytical sensitivity. DETECTR integrates recombinase polymerase amplification (RPA) with Cas12a trans-cleavage, and HPV16/18 DNA recognized by the crRNA triggers reporter cleavage to give a detection limit of 10 copies μL^{-1} as shown in Figure 2A [7]. HOLMES v2 couples loop-mediated isothermal amplification (LAMP) with a thermostable Cas12b module for rapid single-tube DNA and RNA co-detection (Figure 2B) [23]. SHERLOCK combines RPA and T7 transcription with the RNA-guided trans-RNase activity of Cas13a, achieving attomolar detection of viral genomes under optimized pre-amplification conditions (Figure 2C) [15]. A NASBA–CRISPR hybrid uses toehold-mediated switches to convert Cas9 cleavage into a colorimetric readout for bedside Zika virus detection (Figure 2D) [24]. Cas14, activated by phosphorothioate-primed T7 exonuclease processing, selectively interrogates ssDNA targets and initiates downstream signal amplification with minimal components (Figure 2E) [9].

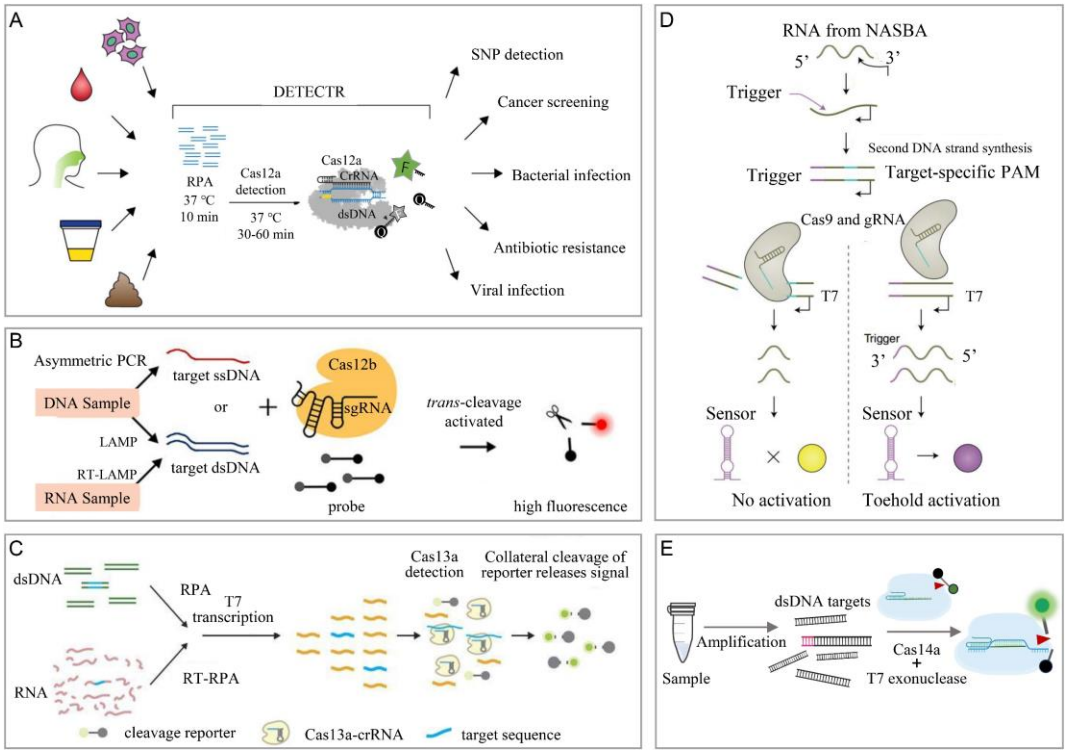


Figure 2. Schematic of CRISPR/Cas molecular diagnostic technology based on nucleic acid pre-amplification. (**A**) Schematic of the CRISPR/Cas12a-based DETECTR methodology for detecting clinical samples in various diagnostic application. (**B**) Schematic diagram of HOLMESv2 detection method. (**C**) Schematic of the SHERLOCK system combined with CRISPR/Cas13. (**D**) Schematic of the NASBACC detection method combining CRISPR/Cas9. (**E**) Schematic of CRISPR/Cas14a for detecting ssDNA.

1.2.2. CRISPR/Cas-Based Molecular Diagnostic Technologies without Nucleic Acid Amplification

Conventional nucleic acid amplification enhances analytical sensitivity but confines diagnostics to sophisticated thermal cyclers, multi-step workflows, and a carryover contamination risk, severely limiting

point-of-care and resource-limited applications. Amplification-free CRISPR assays circumvent these constraints by employing trans-cleavage as an intrinsic signal amplification mechanism [25]. Upon perfect crRNA–target pairing, a single nucleic acid molecule activates the collateral nuclease activity of Cas12 or Cas13, which processively cleaves fluorogenic or electrochemical reporter substrates [26]. Coupling Cas trans-cleavage activity with downstream enzymatic recycling yields exponential signal amplification and routinely achieves sub-femtomolar sensitivity [27]. An archetypal strategy integrates Cas12a with exonuclease III-mediated target recycling, in which target binding initiates iterative cleavage and reduces the detection limit to below 1 fM. Fidelity is further enhanced by rational crRNA engineering, including chemical modifications, locked nucleic acids (LNAs), and optimized quencher spacing [28–32]. Recent transduction schemes further lower the limit; graphene field-effect transistors (FETs) convert Cas13a-directed RNA recognition into conductance changes, enabling label-free RNA quantification at 0.1 pM [33]. Droplet digital microfluidics has eliminated the requirement for pre-amplification altogether. Zhou and colleagues encapsulated Cas13a in picoliter droplets to achieve absolute quantification of single bacterial 16S rRNA copies [34]. Subsequent work leveraged Cas12a trans-cleavage for absolute counting of circulating tumor DNA at 1 copy μL^{-1} in a one-step workflow [35]. Watanabe's SATORI platform integrates Cas13a with micro-well arrays to confine SARS-CoV-2 RNA at 0.1 copies μL^{-1} without enzymatic amplification [36].

Beyond nucleic acids, Collins and colleagues developed CrisprZyme, which combines Cas13 with nanozyme-immunoassays to generate peroxidase-mimicking metal nanoparticle reporters that function at room temperature and are compatible with colorimetric or lateral-flow readouts [37]. The platform supports multiplexed Cas effectors and has been clinically validated for acute myocardial infarction triage and prostate cancer detection, serving as a universal signal amplification module that substantially broadens the target landscape of CRISPR-based diagnostics.

A persistent limitation is the incompatibility of isothermal amplification templates with CRISPR nucleases in single-tube reactions, as trans-cleavage indiscriminately degrades the amplicon. Split-step protocols mitigate this loss but sacrifice simplicity and heighten aerosol contamination. Photocaged guide RNAs resolve this dilemma by enabling light-triggered activation that temporally separates amplification from CRISPR cleavage within a sealed vessel, eliminating contamination while maintaining a single-tube workflow [30,32,38,39].

To approach the ultimate sensitivity limit, CRISPR-based autocatalytic circuits have been engineered to convert a single binding event into an autocatalytic cascade. CALSA (CRISPR/Cas autocatalytic amplification) is a strategy in which LNA-modified split activators initiate a positive-feedback loop in which each Cas12a trans-cleavage event exposes a new activator, achieving a limit of detection of 10 aM for both ssDNA and genomic DNA [40]. Our laboratory further advanced this approach with CONAN (CRISPR/Cas-Only Amplification Network). Here, target dsDNA first activates a canonical Cas12a module whose trans-cleavage displaces an unprotected gRNA; this gRNA then primes a second Cas12a module carrying an auxiliary probe, triggering iterative activator generation and exponential fluorescence increase [41]. Although powerful, such autocatalytic circuits may amplify the background signal if off-target cleavage erroneously initiates the cascade. Rashid Bashir mitigated this risk with a CRISPR/Cas system that embeds two orthogonal Cas12a layers separated by a temperature-gated blocker-linker; kinetic optimization enables attomolar DNA detection in 10 min without pre-amplification [42]. The assay accurately discriminates methicillin-resistant *staphylococcus aureus*, methicillin-sensitive *staphylococcus aureus*, *Escherichia coli*, and HBV directly from blood cultures and implements a multi-input OR gate for syndromic diagnosis. Beyond fluorescence, plasmonic and nanopore transducers are now integrated; nanopore sensors convert Cas13a trans-cleavage activity into ion-current signatures that enable single-molecule RNA quantification. Continued optimization of reaction parameters, digital microfluidic readouts, and hybrid nanomaterial interfaces is establishing a new performance ceiling for amplification-free

CRISPR diagnostics, offering instrument-independent pathogen identification, tumor-mutation tracking, and resistance-gene surveillance at the point of care.

While these platforms share the common principle of CRISPR-mediated signal amplification, their clinical trajectories have diverged considerably (Table 2). Platforms integrating isothermal amplification with lateral-flow detection (e.g., DETECTR, SHERLOCK) have achieved broader adoption, whereas amplification-free approaches, despite eliminating pre-amplification bias, are constrained by sophisticated instrumentation needs.

Table 2. Comparative overview of major CRISPR-based diagnostic platforms.

Platform	Cas Effector	Target	Readout	Key Advantages	Major Limitations	Clinical Status	References
SHERLOCK	Cas13a	RNA	Fluorescence	Attomolar sensitivity; high SNP resolution	Multi-step workflow; initially qualitative	Research and limited clinical use	[15,20,43]
SHERLOCKv2	Cas13	RNA	Multiplex fluorescence	Quantitative and multiplexed detection	Increased system complexity	Early translational stage	[20]
DETECTR	Cas12a	DNA	Fluorescence/ lateral flow	Simplified workflow; strong clinical compatibility	Requires pre-amplification	Advanced toward clinical use	[9,44,45]
HOLMES	Cas12a/Cas12b	DNA/RNA	Fluorescence	High sensitivity and specificity	Amplification-associated bias	Validated in clinical samples	[7,45,46]
Cas12b-based HPV detection	Cas12b	DNA	Fluorescence	Direct detection in human plasma	Pathogen-specific design	Under clinical validation	[46]
Amplification-free CRISPR-SNP chip	Cas–gRNA	DNA	Electrical signal	No amplification; real-time SNP discrimination	High cost; complex instrumentation	Preclinical stage	[47]

2. Precision Diagnostics and Clinical Deployment Scenarios of CRISPR/Cas Molecular Technologies

2.1. Precision Diagnosis of Infectious Diseases

Infectious diseases remain a persistent and emerging threat to global health. Conventional PCR-based pathogen detection provides high analytical sensitivity but is limited in multiplex capacity, making it unsuitable for complex polymicrobial infections or undifferentiated outbreaks. High-throughput sequencing offers comprehensive genomic coverage; however, its high cost, extended turnaround times, and substantial infrastructure requirements hinder rapid population-level deployment.

2.1.1. Rapid Pathogen Screening

The COVID-19 pandemic accelerated the transition of CRISPR-based diagnostics from bench to bedside. In May 2020, Sherlock Biosciences received the first U.S. FDA Emergency Use Authorization for a CRISPR-based SARS-CoV-2 assay built on Zhang Feng's SHERLOCKv2 platform, which multiplexes Cas13, Cas12a, and Csm6 for parallel nucleic-acid detection [48–50]. The original workflow required sequential pipetting and off-cartridge sample processing, increasing contamination risk and turnaround time. Researchers subsequently integrated isothermal amplification and CRISPR-mediated cleavage into a single-tube reaction (STOPCovid.v1) using a thermostable Cas12b variant and a proprietary buffer, reducing hands-on time to less than 1 h [51]. STOPCovid.v2 further compressed the sample-to-answer time to 15 min by incorporating magnetic-bead enrichment into a self-contained microfluidic cartridge, enhancing both analytical sensitivity and clinical utility [50]. This progression from SHERLOCK to

STOPCovid illustrates how iterative engineering can simultaneously improve portability, sensitivity, and operational simplicity, making CRISPR-based diagnostics suitable for resource-limited settings.

To overcome the throughput bottleneck that has long constrained multiplexed pathogen detection, the Sabeti laboratory fused the single-base precision of Cas13 with microfluidic miniaturization to create CARMEN (Combinatorial Arrayed Reactions for Multiplexed Evaluation of Nucleic acids) [52]. The platform interrogates 169 viral sequences within a single sample at femto-molar sensitivity, can be rapidly adapted to emerging pathogens, and decreases reagent costs >100-fold. However, CARMEN's clinical translation was hindered by a large instrument footprint, limited sample capacity, and labor-intensive liquid handling. The team therefore unveiled mCARMEN, a Fluidigm microfluidic cartridge that processes 192 specimens (24-plex) or 96 specimens (96-plex) at less than $13 per test, while automated assay scripting reduces hands-on time to below five hours. Kinetic optimization lifts clinical sensitivity to 98.4% at 10 copies μL^{-1} and compresses turnaround to one hour. By integrating Cas13 and Cas12 modules, mCARMEN delivers absolute quantification of SARS-CoV-2 and influenza viral loads, providing surveillance data seven days ahead of sequencing during the Omicron surge. These iterative advances transform CARMEN from a research prototype into a clinical-grade, high-sensitivity, high-throughput platform that closes the technological gap for multiplex screening, variant tracking, and real-time epidemic response.

2.1.2. Drug-Resistance and Mutation Surveillance

Precision diagnostics enable rapid pathogen identification, yet qualitative or quantitative data alone offer limited guidance in the era of personalized antiviral therapy. Resistance profiling redirects the diagnostic focus from microbial detection to therapeutic prediction by characterizing genotypic resistance mutations, such as HIV integrase inhibitor resistance variants or phenotypic drug susceptibility, as exemplified by *Mycobacterium tuberculosis* drug-susceptibility testing (DST). These approaches guide dynamic and individualized treatment regimens, and CRISPR-based platforms are ideally positioned to close this therapeutic decision loop [53]. A case in point is the *Treponema pallidum* assay devised by Zheng and co-workers at Southern Medical University, which merges PCR with CRISPR/Cas13a to deliver a ten-fold lower limit of detection than conventional RT-PCR and achieves true single-molecule precision [54]. The test achieved 93.3% sensitivity and 100% specificity in a clinical cohort. It has since been extended to strain typing and surveillance of macrolide-resistance mutations, positioning it for post-therapeutic efficacy monitoring.

Similarly, Sabeti's CARMEN architecture has been adapted for HIV drug-resistance surveillance [55]. A microarray of Cas13 effectors, each guided by crRNAs tiled across reverse-transcriptase and *integrase genes*, interrogates key resistance alleles such as *K103N* and *M184V* at single-nucleotide resolution. Using synthetic templates, all six RT and 21 integrase resistance mutations were correctly called with clinical-grade sensitivity and specificity.

When benchmarked against patient samples, CARMEN showed 90% concordance with Sanger sequencing and 86% with next-generation sequencing; an integrated mismatch filter maintains accuracy despite HIV hyper-variability. These features establish CARMEN as a low-cost, high-throughput platform for rapid resistance mapping, providing clinicians with actionable results to tailor antiviral therapy within hours of sample collection.

2.2. Molecular Subtyping of Human Germline Variants and Tumors

Circulating tumor DNA (ctDNA), consisting of fragmented tumor-derived DNA released into peripheral blood, has emerged as a pivotal liquid-biopsy biomarker for non-invasive cancer diagnosis, treatment guidance, and prognostic monitoring [56–58]. Although tissue biopsy remains the histopathological gold standard, its invasiveness, sampling bias, and potential complications preclude serial sampling and dynamic disease tracking. Liquid biopsy offers a powerful non-invasive approach to profiling

tumour genomes; however, the extremely low abundance of ctDNA, typically present at femtomolar concentrations and vastly outnumbered by wild-type DNA, demands analytical platforms that combine attomolar sensitivity with single-base resolution.

To address this challenge, Huo and colleagues at Chongqing University developed an ultrasensitive, label-free CRISPR biosensor that uses Cas12a trans-cleavage to initiate an RNA–DNA chimeric exponential amplification reaction [59]. The resulting G-rich amplicons self-assemble into a dimeric G-quadruplex that binds thioflavin T, yielding more than 100-fold fluorescence enhancement and a limit of detection of 57 aM. The assay is applicable across multiple cancer types and requires only one primer pair per hotspot mutation. Chen and co-workers at Sichuan University proposed an orthogonal, amplification-free strategy by engineering a competitive dual-signal fluorescence sensor [60]. Target ctDNA activates Cas12a, which trans-cleaves a pre-formed G-quadruplex–N-methyl mesoporphyrin IX complex while catalyzing dopamine oxidation to polydopamine, thereby quenching residual NMM fluorescence. The fluorescence decrease scales linearly with ctDNA concentration, enabling marker-free, separation-free quantification with a 4 aM limit of detection within a 40 min workflow for breast-cancer-specific mutations. Both strategies integrate the single-base specificity of CRISPR/Cas12a with the peroxidase-mimicking signal amplification of G-quadruplex structures, eliminating the need for fluorophore–quencher labeling and maintaining robust performance in undiluted serum. The EXPAR-mediated route achieves attomolar sensitivity through multi-turnover catalysis, whereas the competitive-quenching format provides instrument-free operation suitable for low-resource settings. These complementary workflows advance ctDNA analysis to clinical-level sensitivity and portability, offering synergistic options for early cancer detection and real-time therapeutic monitoring.

MicroRNAs, post-transcriptional regulators of gene expression and mediators of intercellular communication, have emerged as critical biomarkers for human disease. Encapsulated within exosomal lipid bilayers, these approximately 22-nucleotide oligomers exhibit exceptional stability and disease-specific enrichment, making exosomal miRNAs prime targets for non-invasive liquid biopsy [61–63]. The gold-standard quantitative RT-PCR workflow, involving RNA extraction, reverse transcription, and pre-amplification, is labor-intensive, prone to sample loss, and poorly suited for point-of-care settings. CRISPR/Cas systems, which combine programmable specificity with built-in signal amplification, now provide an efficient alternative to these constraints [64–66]. Prof. Nie Zhou's group at Hunan University integrated Cas12a into a catalytic hairpin assembly (CHA) circuit to create a dual-amplification engine [67]. Hybridization of the target miRNA to the CHA initiator generates a dsDNA activator that triggers Cas12a trans-cleavage of a fluorogenic reporter, driving the limit of detection into the sub-femtomolar range and enabling multiplexed profiling in a single cuvette. K. Chang and co-workers developed a "Logic-Measurer" cascade that couples a primer-exchange reaction with exonuclease III recycling through an OR gate to quantify miR-21 and miR-375 with a 2.1 fM limit of detection [68]. Evaluation of 315 breast-cancer biopsies achieved 87.3% diagnostic accuracy, underscoring its translational potential. Beyond bulk RNA, Zhang Kaixiang's group delivered CRISPR/Cas13a directly into exosomes via lipid-mediated membrane fusion, enabling *in situ* quantification of exosomal miR-21 in undiluted plasma within 30 min [69]. The assay clearly distinguished breast cancer patients from healthy donors in a pilot cohort, highlighting its potential for real-time therapy monitoring. Collectively, these studies integrate single-base CRISPR precision with diverse enzyme-coupled amplification strategies, surpassing the sensitivity limit of conventional miRNA assays and accelerating the clinical translation of CRISPR-based liquid biopsies [70,71]. While Nie Zhou's CHA circuit achieves superior sensitivity (sub-fM) and multiplexing in a single cuvette, it requires longer incubation times (typically >60 min) compared to Zhang's exosome-targeted delivery (30 min), and lacks the extensive clinical validation (n = 315) demonstrated by Chang's PER-Exo III cascade.

2.3. Multifunctional Sensing of Non-Nucleic-Acid Biomarkers

2.3.1. Protein Biomarker Detection

Accurate quantification of protein biomarkers underpins early diagnosis, treatment stratification, and mechanistic studies. However, conventional enzyme-linked immunosorbent assays (ELISA) are limited by restricted sensitivity, lengthy incubations, and inherent dependence on antibody stability, shortcomings that are amplified when analytes are scarce or matrices are complex. By introducing programmable nucleic-acid recognition and exponential signal amplification into protein detection, CRISPR/Cas systems are reshaping the analytical landscape, enabling label-free immunoassay workflows in which aptamers, nanobodies, or DNAzymes convert protein binding into CRISPR-activating nucleic-acid outputs with attomolar sensitivity.

Early diagnosis of Alzheimer's disease hinges on the precise quantification of Aβ40 and Aβ42 in cerebrospinal fluid. Liu et al. addressed this proteomic challenge with a CRISPR/Cas12a-driven aptasensor, in which Aβ-aptamer binding induces a conformational change that releases a DNA activator, triggering Cas12a trans-cleavage and yielding an ultrasensitive readout of the Aβ42/Aβ40 ratio within minutes. The modular design provides a universal scaffold for any biomarker linked to an aptamer switch [72].

In cardiovascular emergencies, rapid triage of acute myocardial infarction (AMI) demands femtomolar quantification of cardiac troponin I. Zhang and co-workers at Nanjing University developed DATAS-Cas13d (Dual-Aptamer Transcription Amplification Strategy), in which simultaneous binding of two orthogonal aptamers to cTnI nucleates an *in-vitro* transcription module whose RNA output is sensed by Cas13 trans-cleavage activity, achieving 9.3 pM sensitivity in serum at half the cost of conventional ELISA [73]. The assay maintains a LOD of 0.67 ng·mL^{-1} in 10% human serum and discriminates cTnI from interferents proteins such as myoglobin, underscoring its utility in busy emergency departments.

Beyond aptamer-based sensors, CRISPR has been harnessed to monitor enzymatic activity directly. We recently introduced PR-Cas (Protease-Responsive CRISPR), a cascade that couples cancer-associated matrix metalloproteinase-2 (MMP-2) to Cas12a trans-cleavage [74]. An MMP-2-cleavable peptide tethers an RNA polymerase; a single proteolytic event releases the enzyme, triggering multi-copy gRNA transcription that fuels robust Cas12a collateral activity. The platform attains femtomolar sensitivity for MMP-2 in undiluted clinical serum and stratifies patients by metastatic burden, offering a new paradigm for real-time cancer-progression monitoring.

To confront the formidable challenge of quantifying trace proteins in viscous, matrix-rich specimens such as whole blood or sweat, Goldys and colleagues developed CAFI, a fiber-optic immunosensor augmented with CRISPR–Cas12a [75]. In this platform, an antibody–aptamer sandwich captures interferon-γ (IFN-γ), releasing a DNA activator that triggers Cas12a trans-cleavage of a fluorescent reporter. Using only 100 µL of sample, CAFI achieves a limit of detection of 1 fg·mL^{-1} (58.8 aM), representing over 1000-fold improvement compared with conventional ELISA while maintaining picomolar accuracy across undiluted serum, whole blood, and saliva. Swappable capture reagents endow the platform with broad protein versatility, underscoring the modular power of CRISPR-based protein sensing. By integrating aptamer-based sensors, protease-activity reporters, and fiber-optic transduction, CRISPR technology overcomes the traditional limitations of protein detection. Future marriage with hand-held readers, multiplexed biomarker panels, and real-time in-vivo probes will catalyze the clinical translation of CRISPR protein sensors, forging a foundational toolset for precision medicine at the point of care.

2.3.2. Antibody/Metabolite Detection

Accurate quantification of anti-SARS-CoV-2 antibodies is essential for evaluating post-infection immunity, vaccine effectiveness, and protective humoral responses in immunocompromised individuals. Conventional immunoassays, including ELISA and chemiluminescence platforms, suffer from limited

sensitivity and labor-intensive workflows, making them ill-suited for point-of-care deployment [76–81]. More critically, these platforms frequently fail to detect the low-titre antibodies that characterize early infection or the blunted seroconversion observed in immunosuppressed patients after either natural infection or vaccination [79,82–86].

To overcome this analytical gap, Li and colleagues at Sichuan University developed UCAD (Ultrasensitive CRISPR-based Antibody Detection). This system converts antibody binding into a CRISPR/Cas12a-recognizable DNA barcode, achieving attomolar-level sensitivity [87]. The assay harnesses antibody-induced proximity hybridization to form a dsDNA template; after RPA, the amplicon triggers Cas12a trans-cleavage of a fluorogenic or lateral-flow readout. Testing 197 clinical sera (65 from vaccinated donors), UCAD demonstrated 100% sensitivity and 98.5% specificity. Remarkably, in 85 vaccinated kidney-transplant recipients, 85.9% classified as antibody-negative by standard chemiluminescence, UCAD readily quantified low-level antibodies and documented a ≥84.8% seroconversion rate following a third vaccine dose. This capability provides a critical tool for immune monitoring in immunocompromised populations and, as such, constitutes a technical cornerstone that enables both early-phase infection diagnosis and large-scale epidemiological investigations. The Francesco Ricci group has developed diverse DNA-engineered strategies for activating CRISPR/Cas12a signaling, including PAM-engineered toehold switch-mediated strand displacement reactions, synthetic antibody–DNA conjugates for direct activation, and the MAIGRET (Molecular Assay based on antibody-Induced Guide-RNA Enzymatic Transcription) system, which collectively enable the transduction of antibody–antigen recognition events into Cas enzyme collateral cleavage signals. These approaches significantly expand the scope of CRISPR-based detection beyond nucleic acids, enabling femtomolar-to-picomolar sensitivity for various targets, including antibodies (e.g., anti-digoxigenin, anti-HA, and Cetuximab), protein antigens (e.g., SARS-CoV-2 spike protein, EGFR, and MUC1), and small molecules (e.g., digoxigenin) in complex biological matrices [88–90].

Accurate quantification of small molecule metabolites such as uric acid and glucose is central to the management of gout, diabetes, and related chronic disorders. Conventional approaches, such as high performance liquid chromatography or invasive blood sampling followed by benchtop analysis, limit the feasibility of point-of-care testing. Zhang and co-workers addressed this limitation with CaT SMelor, an allosteric transcription factor (aTF) gated CRISPR sensor that converts small molecule binding into an amplifiable DNA output [91]. Uric-acid-mediated conformational switching of the aTF licenses RPA amplification of a Cas12a activator; subsequent trans-cleavage of a fluorogenic reporter yields a 10 nM limit of detection that correlates seamlessly with HPLC and clinical chemistry analyser readouts across 32 patient sera. The reagent cost (less than fifty cents per test) and ambient-temperature operation position CaT-SMelor as a field-forward alternative for resource-limited settings.

Building on this concept, a non-invasive CRISPR biosensor has been designed for painless glucose surveillance in tears and saliva [92]. Glucose oxidase (GOx) oxidizes glucose to gluconolactone with stoichiometric H_2O_2 release; the peroxide triggers self-cleavage of a pistol-like DNAzyme (PLDz), liberating a DNA fragment that activates Cas12a trans-cleavage of a quenched reporter. This cascade achieves 0.1 μM sensitivity, significantly outperforming conventional electrochemical glucose sensors while eliminating the need for finger-stick blood sampling. Collectively, these metabolite-to-DNA transducers exemplify how CRISPR/Cas systems can make chronic-disease monitoring accessible through amplification free handheld workflows.

CRISPR-based biomarker detection follows a universal design principle in which a molecular recognition element, such as an antibody, an allosteric transcription factor, a DNAzyme, or its synthetic analogue, converts a non-nucleic acid target into a programmable DNA cue that triggers the trans-cleavage cascade of CRISPR/Cas. UCAD initiates DNA probe hybridisation through antibody–antigen proximity, whereas CaT-SMelor uses ligand-induced conformational changes in an aTF to release a DNA template,

and the glucose sensor links enzymatic H_2O_2 production to DNAzyme self-cleavage to furnish the required activator. This modular architecture retains attomolar sensitivity and single-base specificity across analyte classes and remains independent of the upstream recognition modality.

Developing next-generation affinity reagents such as aptamers, *de novo* protein binders, and artificial receptors will expand CRISPR diagnostics to tumour antigens, environmental toxins, and other emerging biomarkers. Concurrent integration with lateral-flow strips, microfluidic cartridges, and handheld readers should translate these molecular advances into household-ready devices suitable for resource-limited settings, establishing a versatile technological platform for precision medicine and global health surveillance.

In addition to clinical applications, CRISPR/Cas diagnostics are increasingly deployed in agriculture and environmental monitoring. Multiplex RT–RPA–Cas12 panels detect *potato virus X*, *potato virus Y*, wheat stripe rust, and *Magnaporthe oryzae* in a single sealed tube [93–98]. Attomolar sensitivity and single-base specificity are exploited to scan GMO inserts in food and feed, discriminate SNPs linked to herbicide resistance, and trace yield-determining loci in crops [99]. Environmental deployments extend the same molecular logic to quantify viral and bacterial load, heavy-metal ions, antibiotic residues, and pesticide contaminants in water, soil, and aerosol samples. These cross-sector successes illuminate a versatile future for CRISPR/Cas molecular diagnostics stretching from clinic to crop and riverbank to factory floor.

3. Future Perspectives on CRISPR-Based Diagnostics

3.1. Innovations in Miniaturization and Integration Technologies

The future development of CRISPR-based diagnostic technologies is expected to focus on miniaturization, high-throughput detection, and interdisciplinary integration, aiming to overcome current technological limitations and expand application scenarios. Miniaturization and portability represent key directions in the evolution of CRISPR/Cas diagnostic systems [100]. As an integrated platform, microfluidic chips adopt an integrated design concept that consolidates sample pretreatment, nucleic acid amplification, and CRISPR/Cas-based detection within a single device, thereby achieving complete automation and miniaturization. Fingernail-sized chips, for example, extract and detect HIV nucleic acids directly from whole blood [101,102]. Concurrently, smartphone-integrated readers and wearable form factors propel the field toward an “instrument-free” paradigm. The use of low-energy Bluetooth or Wi-Fi for streaming signals from various detection modalities (colorimetric, fluorometric, amperometric) to a mobile processor enables real-time, multi-modal image deconvolution via convolutional neural networks. This system minimizes hardware overhead and facilitates diagnostics in resource-limited environments [103].

Li and colleagues developed CLIPON (CRISPR and Large DNA assembly Induced Pregnancy strip for signal-ON detection), a portable platform that repurposes commercial pregnancy dipsticks for wash free, visual detection of both single and double stranded DNA in homogeneous solution [104]. A dedicated smartphone application and disposable microfluidic chip convert the visual readout into a quantitative result, delivering a fully portable assay. To maximize simplicity, Wang and collaborators developed SCOPE (Streamlined CRISPR On Pod Evaluation), a single-step platform that compresses sample-to-answer time to 12 min [105]. Rapid chemical lysis liberates viral nucleic acids from rash exudate, oral swabs, saliva, or urine within 2 min, followed by a one-pot RPA-Cas13a reaction that achieves *monkeypox virus* detection in 10 min. All steps, lysis, amplification, CRISPR cleavage, signal acquisition, and automated interpretation, which are executed on the palm-sized CPod device, offering a proper sample-in/answer-out solution for field diagnostics.

Wearable and real-time pathogen surveillance is transitioning from laboratory prototypes to a daily-life necessity. Collins and colleagues have seamlessly interwoven CRISPR chemistry into a 3-g, freeze-dried, cell-free synthetic-biology sensor that retains laboratory-grade sensitivity and specificity while embedded in everyday textiles [106]. Laminated within the fabric of an N95 respirator, the device passively

captures SARS-CoV-2 aerosols, rehydrates on exhaled moisture, and completes a colorimetric Cas12 readout within 90 min without requiring an external power source. Encapsulated in soft silicone elastomers or woven fibres, the sensor continuously logs exposure events and transmits time-stamped data via low-energy Bluetooth to a smartphone gateway. By fusing micro-scale CRISPR reactors with ultra-low-power circuitry and cloud analytics, the platform compresses sample-to-answer time to under 30 min. It establishes a three-tier surveillance network spanning individual, community, and clinical levels, delivering instantaneous, high-resolution intelligence for outbreak containment, chronic-disease management, and personalized health tracking.

Amid the global COVID-19 pandemic, microfluidic technology has significantly advanced the performance of point-of-care testing (POCT) of SARS-CoV-2 assay through synergistic integration with nucleic acid analysis, immunoassays, and electrochemical biosensing platforms [107–112]. As a versatile multimodal platform, microfluidics accommodates nucleic-acid amplification, antigen-antibody recognition, and optoelectronic signal transduction within a miniaturized, automated framework. Notably, CRISPR-based microfluidic platforms, by precisely coupling molecular recognition with robust signal amplification, have substantially enhanced detection sensitivity and broadened the diagnostic scope [113,114].

Compared with conventional diagnostic workflows, microfluidic systems offer several advantages, including reduced reagent consumption, streamlined automation, and minimized risk of cross-contamination. Customized microfluidic designs have also addressed critical technical challenges associated with CRISPR-based assays [115]. For instance, paper-based microfluidic devices enable simple, colorimetric visual readouts, significantly simplifying assay procedures [116]. Enclosed microfluidic cartridges effectively prevent aerosol leakage, enhancing biosafety during testing [117–120]. Centrifugal or electrochemically integrated modules can consolidate nucleic acid extraction, purification, and detection into a single, standardized workflow [121,122]. Furthermore, by precisely manipulating discrete droplets, digital microfluidics enables single-molecule-level absolute quantification, pushing the limits of analytical sensitivity [123,124].

Current research efforts are increasingly focused on identifying and optimizing microfluidic architectures that align with the specific methodological requirements of different detection modalities, aiming to maximize automation, reproducibility, and diagnostic performance. This endeavor requires in-depth innovation, including microchannel topology design, fluidic control systems, and system-level engineering integration. In the future, CRISPR/Cas-powered microfluidic diagnostics, through interdisciplinary convergence, are poised to establish transformative technological paradigms in pathogen detection at the point of care, antimicrobial resistance gene screening, and precision medicine. These advances are expected to drive the evolution of *in vitro* diagnostics toward greater portability, intelligence, and networked connectivity, ultimately reshaping the landscape of global health diagnostics.

3.2. High-Throughput and Intelligent Diagnostics

As CRISPR diagnostic technology gradually advances toward clinical-grade testing, achieving high-throughput, multi-target analysis, and intelligent result interpretation has become a central direction for future development. Regarding multiplex detection, the CARMEN platform integrates Cas12/Cas13 hybrid systems with fluorescence-barcoded microfluidic chips to simultaneously identify 169 pathogens in a single reaction. Droplet microfluidic sorting technologies, such as Drop-CRISPR, partition samples into millions of microdroplets and, combined with the trans-cleavage activity of Cas proteins, enable single-molecule counting and multi-target genotyping, thereby markedly enhancing throughput and sensitivity. With the continuous development of artificial intelligence, especially breakthroughs in machine learning, CRISPR/Cas systems are shifting from basic research tools to precision, intelligent molecular diagnostic platforms, opening new avenues for innovation. Zhang Feng's team developed the machine-learning model CRISPRscan, which predicts crRNA–target binding efficiency and off-target risk, guiding the design of

high-affinity crRNAs and improving detection sensitivity by more than tenfold. Youchun Xu's group at Tsinghua University proposed "mutaSCAN", an extraction-free mutation-detection strategy [125] that combines sensitive RT-LAMP amplification with specific CRISPR/Cas12a detection in a single microfluidic chip unit. This approach uses an innovative lyophilization strategy to resolve temperature compatibility between the two steps and successfully integrates variable-throughput microfluidic chips. In addition, the workflow incorporates AI algorithms and user-friendly imaging devices to achieve high-throughput and automated result reading. mutaSCAN can detect 96 samples within 30 min at a sensitivity of 250 copies mL^{-1}, enabling direct detection of SARS-CoV-2 and its variants from patient samples. The method demonstrates an ultrahigh-throughput analysis system using microfluidic multiwell plates, achieving rapid and accurate detection of SARS-CoV-2 and its variants with no false positives in negative samples. The research team also improved detection versatility and field applicability by developing a lysis buffer and optimizing microfluidic chip design. It achieved on-site automated result interpretation through AI-based image recognition algorithms. Clinical sample testing showed that mutaSCAN provides high positive and negative predictive concordance. Furthermore, synergistic innovation between bioinformatics and CRISPR technology offers a new approach for pathogen tracing. The deep integration of high-throughput and intelligent features significantly enhances the throughput, sensitivity, and accuracy of CRISPR diagnostics. It lays a solid foundation for its broad application in clinical medicine, disease surveillance, and public health [126–130].

Compared with other detection methods, CRISPR systems exhibit distinct advantages. First, owing to their high specificity and sensitivity, CRISPR systems achieve single-base resolution through precise crRNA–target nucleic acid pairing, enabling accurate discrimination between conserved pathogen sequences and host gene mutations. Second, CRISPR systems possess strong programmability and multifunctionality, allowing customized detection of diverse pathogens and biomarkers to meet diversified and personalized diagnostic needs. Their integrated detection capability also makes "sample-in, answer-out" automated workflows feasible, reducing cumbersome operational steps and lowering contamination risks. In addition, integrating CRISPR systems with artificial intelligence endows them with intelligent data-processing capacity, enabling rapid and accurate test results analysis. These advantages in specificity, sensitivity, programmability, integrated detection, and AI compatibility position CRISPR systems as powerful tools for high-throughput and intelligent diagnostic applications. As cross-disciplinary technologies advance, CRISPR diagnostic platforms are expected to evolve into intelligent molecular diagnostic systems that integrate detection, analysis, and decision-making.

3.3. Core Challenges in Clinical Translation

CRISPR-based diagnostics have demonstrated exceptional performance in research settings, yet their transition to the clinic remains constrained by a series of upstream bottlenecks, foremost among them the absence of a standardized, high-fidelity sample-preparation pipeline [25]. The extraction efficiency and purity of cell-free nucleic acids, such as circulating tumor DNA and viral RNA, directly influence detection sensitivity. However, extraction approaches such as magnetic bead-based and column-based methods show limited capacity to enrich low-abundance targets, resulting in suboptimal recovery rates. Moreover, heterogeneous pre-processing requirements for diverse specimens, including sputum, feces, and tissue sections, necessitate the development of universal lysis reagents and automated platforms. Nonetheless, most current CRISPR-based assays still rely on manual operations, thereby introducing variability in reproducibility.

Beyond sample-to-answer integration, the credibility of absolute quantification remains an equally formidable bottleneck. Digital CRISPR formats, exemplified by droplet-microfluidic single-molecule counting, achieve a limit of detection of 1 copy μL^{-1}, yet they demand high-precision syringe pumps, high-numerical-aperture optics, and defect-free microfluidic masters, pushing instrument costs beyond 50,000 dollars, a price point incompatible with district hospitals or low-resource settings. The Quake-group dPCR–

CRISPR hybrid reduces the hardware bill of materials to approximately 5000 dollars, but the long-term drift of the mandatory standard curve is still unresolved. Inter-batch variation in droplet volume, surface fluorophore adsorption, and thresholding algorithms routinely produces more than 25% relative standard deviation (RSD) in quantitative calls.

The core components of CRISPR-based assays include Cas proteins and crRNA, which must be stored at low temperatures to maintain activity and ensure the reliability of test results. Although lyophilization can extend their shelf life, Cas protein activity typically declines after rehydration, reducing detection accuracy. Because Cas proteins are susceptible to temperature, future studies could explore optimized storage conditions and reaction buffers, or the addition of protein stabilizers to prevent denaturation and degradation, thereby enhancing the stability and reliability of diagnostic systems [131]. In addition, the large-scale synthesis cost of crRNA far exceeds that of traditional PCR. Delays in regulatory approval and clinical validation further exacerbate the challenges of commercialization. Confronted by intertwined technical and economic bottlenecks, the field is turning to convergent, cross-disciplinary innovation as the most credible escape route. Systematic delays in navigating regulatory approval (averaging 18–36 months for novel biochemistry) and the absence of standardized clinical validation protocols further exacerbate commercialization challenges. A flagship example is mutaSCAN: a Cas12a-gated, high-throughput microfluidic platform that merges one-pot RT-LAMP amplification with convolutional-neural-network (CNN)-driven image analytics to deliver 96-plex results in 30 min. The system retains an analytical limit of 250 copies mL^{-1} across diverse clinical matrices while simultaneously profiling emergent mutations in the same workflow.

Equally instructive is the generative-design strategy introduced by Hayden C. Metsky and co-workers [132]. By retrofitting the ADAPT pipeline with a generative adversarial network coupled with an evolutionary algorithm, they established an in silico design environment that enables unconstrained exploration of crRNA sequence space, transcending the finite repertoire of naturally occurring viral genomes. The resulting software suite outputs synthetic guide sequences that intentionally harbour engineered mismatches yet preserve on-target activity. In head-to-head comparisons, these artificial crRNAs tripled the limit of detection relative to wild-type counterparts. They discriminated single-nucleotide variants of SARS-CoV-2 with 98% concordance against next-generation sequencing, thereby setting a new benchmark for precision CRISPR diagnostics.

CRISPR/Cas-driven molecular diagnostic technologies are rapidly moving from laboratory research to clinical application, with their core breakthrough being the realization of accurate “sample-in, answer-out” testing that meets the universal demand for equipment-free and rapid results [133,134]. Integrating nucleic acid isothermal amplification modules with CRISPR/Cas molecular recognition elements allows these platforms to screen multiple pathogens in complex biological samples without sophisticated instruments. Synergistic innovations in synthetic biology and artificial intelligence further enhance assay performance. Deep-learning-based crRNA design algorithms markedly improve the target specificity of Cas proteins, while Cas variants produced through directed evolution can elevate detection sensitivity to the single-copy level. Because of their inherently visual signal output, CRISPR biosensors show unique advantages in primary healthcare and at-home self-testing. Platforms built on lateral-flow strips, using colloidal gold nanoparticles or fluorescent probes, provide dual-mode colorimetric and fluorescence readouts, thereby overcoming the traditional dependence of molecular diagnostics on specialized instruments [135–138]. Clinical validation data show that home self-test strips for influenza A/B and *Helicobacter pylori* achieved more than 90% concordance in multicenter trials, and participants following standardized procedures could determine infection status within 15 min. This combination of operational simplicity and result reliability drives precision diagnostics toward a distributed model of community-based prescreening and at-home testing. With their visual and portable advantages, CRISPR test strips are well-suited for primary healthcare

scenarios, and their complementary integration may create a complete POCT system covering community screening and precise pathogen subtyping.

The cross-integration of CRISPR/Cas biosensors with cutting-edge technologies such as nucleic acid design, nanomaterials, biochips, and artificial intelligence is opening new possibilities for molecular diagnostics and enabling the development of more efficient, intelligent, and versatile POCT devices. This convergence drives the evolution of next-generation diagnostic tools and lays the foundation for rapid molecular testing within POCT applications. To realize the full potential of CRISPR diagnostics in clinical practice and global precision medicine, it is essential to overcome persistent technical, economic, and regulatory bottlenecks. Otherwise, these constraints will significantly hinder the widespread application of CRISPR-based diagnostic technologies.

4. Conclusions

CRISPR–Cas systems have catalysed a paradigm shift in molecular biology and now stand at the forefront of next-generation diagnostics, poised to transform pathogen detection, genetic-variant profiling, and patient-tailored medicine [139,140]. Yet for all their promise, current assays still fail to converge on the quartet of clinical imperatives—high throughput, single-molecule sensitivity, pocket-sized hardware and dollar-scale unit costs. Therefore, a dispassionate audit of these persistent bottlenecks (Figure 3) and a roadmap for their future dissolution are indispensable to accelerate the bench-to-bedside journey of CRISPR-based diagnostics and unlock its full theoretical and practical potential [141–143].

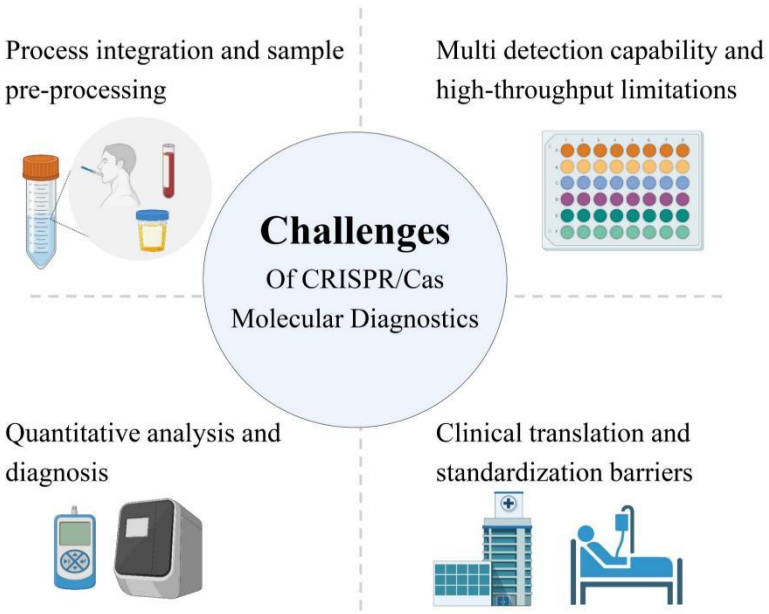


Figure 3. The Challenges of the CRISPR/Cas system in molecular diagnosis.

Foremost among the hurdles to clinical translation is the imperfect specificity of CRISPR diagnostics. However, Cas effectors can be programmed through crRNA–target complementarity, complex biological matrices still yield measurable trans-cleavage [144,145]. The problem is exacerbated when discriminating near-identical pathogens or interrogating single-nucleotide variants, where a trans-cleavage base mismatch can tilt the balance between a true positive and a false positive call [146,147]. Dual gating recognition circuits, seed trimmed crRNAs, and high fidelity Cas variants have all shown promise in proof of concept studies, yet none have advanced beyond retrospective cohorts. Therefore, a rigorously validated, design rule based toolkit for suppressing mispriming and background noise remains an unmet and rate limiting clinical need.

Secondly, streamlining detection workflows and standardizing sample preprocessing are pivotal for translating CRISPR/Cas systems into practical point-of-care diagnostics. While the ideal molecular diagnostic platform would enable a "one-step" workflow, most current CRISPR-based assays still depend on a multi-step process involving nucleic acid extraction, amplification, and signal readout. These systems are commonly coupled with isothermal amplification techniques such as RPA or LAMP to achieve clinically relevant sensitivity. However, this multistage approach increases operational complexity and raises the risk of cross-contamination, limiting the feasibility of rapid deployment in resource-limited or field settings [148,149]. To overcome these challenges, future research should prioritize the development of highly integrated platforms such as microfluidic-based, fully enclosed detection chips or *in situ* reaction systems that eliminate the need for nucleic acid extraction, thereby advancing the realization of a true "sample-to-result" diagnostic paradigm.

Quantitative inadequacy remains a cardinal bottleneck constraining the clinical translation of CRISPR–Cas molecular diagnostics. Predominant end-point formats offer only binary or semi-quantitative readouts. They are blind to reaction kinetics, precluding precise viral-load monitoring, treatment-response surveillance, or any application requiring copy-number resolution. Coupling Cas-based assays with real-time fluorescence tracking, electrochemical transduction, or digital-droplet partitioning is an urgent priority; such integrations promise next-generation platforms that are simultaneously quantitative, high-throughput, and automation-compatible.

Multiplexing and massively parallel processing must evolve in parallel. Divergent cleavage kinetics among Cas orthologs, and the narrow reaction windows demanded by orthogonal targets, frustrate simultaneous multi-analyte detection. Although proof-of-concept strategies such as SHERLOCKv2, CARMEN, and their microfluidic-array, bar-coded, or spatially segregated progeny have demonstrated impressive scalability, they remain dependent on complex instrumentation and labor-intensive workflows, which are incompatible with decentralized testing. A convergent engineering agenda that unifies reaction chemistry, normalizes signal output, and standardizes readout modalities is thus essential if multiplex CRISPR diagnostics are to graduate from specialist laboratories to routine clinical use.

Notwithstanding the hurdles described above, the long-range outlook for CRISPR/Cas molecular diagnostics remains unequivocally promising. Evolutionary momentum is already evident, with an expanding arsenal of newly annotated Cas effectors differentiated by mass, cleavage mechanism, PAM plasticity, and thermal tolerance. This diversity has broadened the engineering design space, providing unprecedented freedom to develop lighter, faster, and more robust assays. Concurrently, machine-learning algorithms trained on million-sequence datasets are permeating every stratum of the development pipeline, accelerating guide-RNA optimisation, in-silico off-target filtering, kinetic modelling, and automated signal triage, while simultaneously shrinking user intervention and cycle time.

Beyond analytical performance, the strategic value of CRISPR diagnostics is becoming indelible. Attomolar sensitivity, minute footprint, and minimal power requirements converge to enable outbreak interception, personalised therapy titration, and decentralised testing in low-resource theatres. The COVID-19 pandemic provided an unplanned but decisive validation: field-forward CRISPR prototypes repeatedly delivered laboratory-grade sensitivity and specificity within 30 min, surpassing gold-standard RT-PCR in logistical agility and time-to-action, with speed translating directly into outbreak containment. Prospectively, more profound symbiosis with nanomaterials science, micro/nanofluidics, flexible electronics, and cloud-connected analytics is poised to engender a continuum of sensing ecosystems. These next-generation systems will evolve beyond episodic testing to function as continuous health monitors. By integrating real-time molecular surveillance, AI-powered early-warning algorithms for pathogen threats, and closed-loop therapeutic feedback, they will reposition CRISPR diagnostics from a reactive tool to a foundational component of proactive, precision public health.

In conclusion, CRISPR/Cas diagnostics have vaulted to the forefront of molecular medicine by uniting attomolar sensitivity, single-nucleotide specificity, and field-deployable simplicity in one cohesive platform. Future assays should balance sensitivity with cost and speed, as many diagnostic decisions do not benefit from attomolar limits. Ongoing engineering advances, driven by cross-disciplinary efforts, are expected to overcome existing challenges and enable these assays to transition from research tools to clinical standards. This will ultimately establish them as key components in global health security and precision therapeutics.

Statement of the Use of Generative AI and AI-Assisted Technologies in the Writing Process

During the preparation of this manuscript, the author(s) used Kimi and DeepSeek in order to assist with language editing and manuscript polishing. After using these tools, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the published article.

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Author Contributions

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