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CRISPR-based diagnostics for circulating cell-free DNA: a paradigm shift in precision oncology

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Circulating cell-free DNA (cfDNA) has been established as a minimally invasive liquid biopsy biomarker with utility in the diagnosis of cancer, monitoring of treatment response, and detection of minimal residual disease. The clinical utility of cfDNA is currently constrained by the low abundance of circulating cfDNA fragments, high fragmentation rates, and short half-life, making it technically challenging to detect in a patient sample. Current molecular approaches for cfDNA detection, including ddPCR and NGS, are time-intensive, expensive, and unsuitable for low-resource settings and point-of-care testing. The CRISPR-Cas system offers a novel and operationally simple approach to cfDNA detection by being single nucleotide specific and compatible with isothermal and amplification-free workflows. In this review, we discuss CRISPR-based assays for cfDNA, beginning from Cas9 enrichment-type assays to promising collateral cleavage platforms employing Cas12a and Cas13a that have countered traditional bottlenecks concerning diagnostic testing. We also provide a comparative analysis of the emerging platforms for key cancer mutations with a discussion around translational scope, including implications from CRISPR-based diagnostic patents. The convergence of sensitivity, speed, multiplexing, and microfluidic integration of CRISPR diagnostics will undoubtedly constitute a next-generation approach for cfDNA analysis, presenting a great promise in impacting precision oncology and increasing access to cancer diagnostics across low-resource settings.

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1. Introduction

Cancer stands as the second leading cause of death globally, posing serious public health threats.[1] While early detection significantly improves treatment outcomes and five-year survival rates, effective cancer management requires more than just early diagnosis.[2] Steady improvement in a patient's survival is equally contingent on tracking evolving genomic changes in cancer cells, along with the detection of minimum residual disease,[3] drug resistance, and cancer relapse.[4] Traditional tissue biopsies are still deemed the best practice for diagnosing cancer; however, they fall short of addressing these evolving clinical demands. Their invasive nature of sampling, site-restricted sampling, and lack of consideration of multifaceted intra-tumor heterogeneity obstruct real-time monitoring and comprehensive tumor profiling.[5] Also, their frequent implementation is impractical and incredibly burdensome for patients. Within this framework, liquid biopsy (LB) has surfaced as a minimally invasive, repeatable, and clinically informative alternative. LB refers to the collection and subsequent molecular evaluation of non-solid biological tissues, predominantly blood, but also encompasses urine, saliva, and cerebrospinal fluid. It facilitates the capture of tumor-derived materials such as circulating tumor cells (CTCs), extracellular vesicles (EVs), and circulating cell-free DNA (cfDNA), thereby providing real-time perspectives on tumor development and its evolving genetic landscape.[6] Out of these components, cfDNA has garnered particular attention for its clinical relevance.

cfDNA refers to fragments of DNA released into blood circulation as a result of cell necrosis or apoptosis. A small subset of cfDNA known as circulating tumor DNA (ctDNA) specifically comes from dying tumor cells and carries highly informative characteristics.[7] Since ctDNA contains specific alterations of a tumor's genome, such as point mutations and other changes in methylation patterns, it serves as a molecular snapshot of the tumor tissue.[8] For instance, in cancers caused by HPV, its viral DNA is integrated into the host genome, and these infected DNA fragments are released into circulation upon cell death, which can be targeted for minimally invasive HPV testing.[9] Additionally, ctDNA from tumors with driver mutations like EGFR, KRAS, and BRCA-1 subsequently can be found in plasma, allowing for genotyping that paves the way for personalized treatment plans.[8] Regulatory approvals confirm the clinical benefits of ctDNA and endorse using plasma-derived DNA for non-invasive diagnosis and monitoring of treatment response. This is supported by the FDA's approval to implement the SEPT9 methylation test for colorectal cancer and EGFR mutation testing for NSCLC, certified by the European Medicines Agency (EMA).[10] Furthermore, research proved that ctDNA is capable of earlier detection of relapse compared to radiological imaging,[11] predicting shorter overall and disease-free survival time when positive after treatment,[12] and dominating adjuvant therapy decision-making.[13] Additionally, ctDNA analysis aids in monitoring treatment responses, tracking clonal evolution, and identifying mutations associated with acquired resistance.[7,8]

Despite such benefits, the implementation of ctDNA testing is finite in clinics, as ctDNA detection is technically challenging, owing to numerous inherent biological limitations. ctDNA is highly fragmented, found at very low abundance (0.1–10% of total cfDNA), and has a short half-life (~2 hours), which limits its detectability in early-stage disease or minimal residual states.[14] This low signal-to-noise ratio, caused by dilution in a background of wild-type cfDNA, necessitates highly sensitive, specific, and robust detection assays.[15] Emerging molecular technologies such as digital droplet PCR (ddPCR) and next-generation sequencing (NGS) have made considerable progress in cfDNA analysis.[16] However, they are expensive, equipment-intensive, time-consuming, and often lack the sensitivity needed to detect ultra-low copy variants, especially during early-stage disease. In addition, the expense and technical sophistication of such technologies restricts their application to low-resource facilities or point-of-care (POC) settings. To tackle these drawbacks, scientists have turned to the CRISPR-Cas system. This tool, first created to edit genomes, now plays a major role in molecular testing. Owing to its flexible target-specific sequence programming and ability to spot even a single base difference, CRISPR-Cas offers a promising alternative to detect ctDNA.[17] Its adaptability alongside isothermal amplification methods, compatibility with fluorescence or lateral-flow readouts, and potential for miniaturization make it an ideal candidate for next-generation liquid biopsy-based diagnostic platforms (Fig. 1).[18] This review highlights how CRISPR-based assays are transforming the era for the detection of circulating cell-free DNA, with a particular focus on their innovations to overcome current shortcomings, their comparative advantages, and future potential in advancing cancer diagnostics.

2. Overview of the CRISPR-Cas system

CRISPR (clustered regularly interspaced short palindromic repeats) was first discovered as an adaptive immune mechanism, present in archaea and bacteria, that offered cellular protection against invading foreign genetic material.[19] This system can be understood in three phases: (1) adaptation, in which fragments of foreign DNA are inserted as "spacers" into the host genome; (2) expression, where the CRISPR array is transcribed and processed into CRISPR RNAs (crRNAs); and (3) interference, during which crRNAs direct CRISPR associated (Cas) proteins to complementary sequences in the target DNA or RNA for cleavage at a site-specific manner.[20] Despite underlying similarities, Cas proteins are classified typically into two classes. Class I systems depend on multi-component multi-protein effector complexes, whereas class II systems are considered more favorable for diagnostic applications as they rely on one multi-domain Cas protein. For class II systems, three effector proteins, Cas9 (type II), Cas12 (type V), and Cas13 (type VI), have been widely repurposed for nucleic acid detection and each confers distinct properties that are now leveraged across multiple CRISPR-based diagnostics (Table 1).[21]

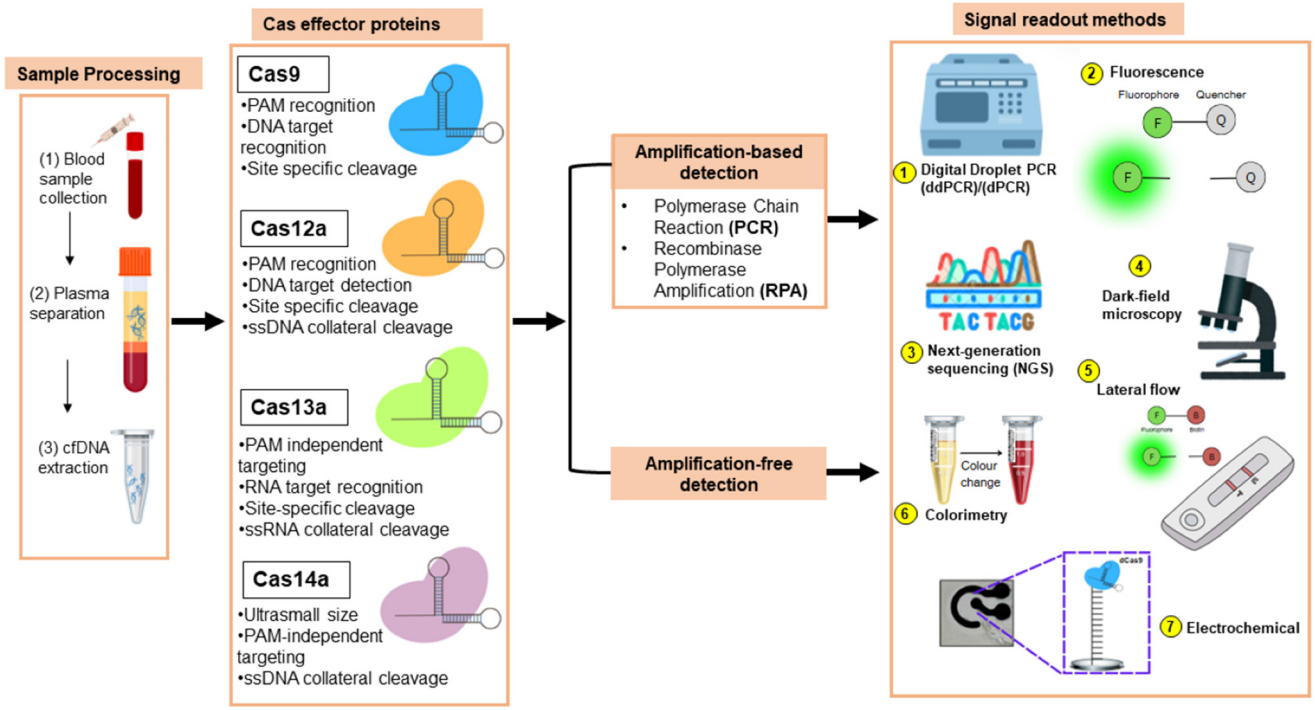

Fig. 1 Overview of the basic mechanism involved while adopting CRISPR for cfDNA diagnosis, starting from sample processing, Cas effector-based target cleavage, amplification-assisted or amplification-free detection assay, and the final signal readout methods.

Cas9 is a double-stranded DNA endonuclease that requires a 5′-NGG-3′ protospacer adjacent motif (PAM) for target recognition and delivers precise double-strand breaks at the target location through a single guide RNA (sgRNA), the fusion of crRNA and tracrRNA.[22,23] While wild-type Cas9 is useful for targeted cleavage, its catalytically inactive variant, dead Cas9 (dCas9), does not exhibit endonuclease activity but is a sequence-specific DNA-binding protein. This property allows dCas9 to be adopted as a case for use in biosensors where it serves as a molecular anchor in label-free electrochemical systems to provide a precise detection method without nucleic acid cleavage.[24] Cas12a (formerly Cpf1) is used to recognize a T-rich PAM (5′-TTTN-3′) and, after activation, carries out dual activities—cleaving target double-stranded DNA, and also triggering the collateral cleavage of nearby single-stranded DNA (ssDNA).[24] This *trans*-cleavage activity underlies the basis of sensitive signal amplification in CRISPR diagnostics.[21] Cas12a is an important component of the DETECTR (DNA endonuclease targeted CRISPR trans reporter) platform which was one of the first CRISPR diagnostic systems targeting human papillomavirus (HPV). Within DETECTR, target DNA is first amplified using recombinase polymerase amplification (RPA), and then after target-specific binding occurs, Cas12a is activated to cleave a fluorophore-quencher reporter molecule, releasing a fluorescence signal. The DETECTR system demonstrated attomole-level sensitivity and was key in depicting the utility of CRISPR for DNA diagnostics.[25] Cas13a, in contrast, specifically targets single-stranded RNA (ssRNA) and lacks DNase activity. Once the Cas13a–crRNA complex recognizes its RNA target, it activates collateral cleavage of surrounding ssRNA molecules.[26] This property underpins the SHERLOCK (specific high sensitivity enzymatic reporter unlocking) platform, the earliest system to use CRISPR for RNA-based diagnostics. SHERLOCK combines Cas13a enzymology with isothermal amplification and signal transduction *via* fluorescent or lateral flow reporters, enabling the detection of RNA viruses or circulating tumor RNA (ctRNA) with single-nucleotide specificity. The platform is highly sensitive to detecting targets at attomolar concentrations and is highly suitable for field-deployable testing.[27] Unlike Cas12a, this protein can also tolerate a broader target range, as Cas13a does not require PAM sequences for activation.[28] DETECTR and SHERLOCK together represent the first CRISPR-based diagnostic platforms comprising two different Cas proteins to offer programmable enzymatic tools for precise and sensitive nucleic acid detection. They laid the groundwork for a flurry of CRISPR diagnostic innovation that now employs amplification-free formats, nanomaterials, and/or electrochemical systems, marking a shift to portable, rapid, and low-cost diagnostics suitable for clinical translation in precision oncology.

Apart from these commonly used Cas enzymes, there exist other Cas proteins that are not widely explored for targeting cfDNA for cancer diagnosis. Cas14 protein is yet another Cas enzyme which is the smallest of all Cas enzymes in class II often falling in the range of 400 to 700 amino acids. Cas14 enzymes may be small, but they have substantial cleavage activities mirrored in Cas12, action on *cis* double-stranded DNA (dsDNA) and *cis*/*trans* single-stranded DNA (ssDNA). The

Table 1 Studies employing CRISPR-Cas systems for cfDNA-based cancer detection

S. no.	Method/platform	Cas protein	Amplification used	Target mutation/cancer type	Mechanism or modification	Sensitivity/LOD	Key advantage	Sample type	Ref.
1	Pre-amplification mutant enrichment	Cas9	PCR	EGFR exon 19 deletion	Pre-PCR cleavage of wild-type and mutant enrichment	0.01% allele frequency	Improves allele detection by enriching the mutant	Plasma cfDNA	29
2	CRISPR-CPPC	Cas9	PCR	EGFR T790M (NSCLC)	Post-PCR mutant enrichment	93.9% sensitivity, 13-fold allele freq. gain	Works on fragmented cfDNA, better than ddPCR alone	Plasma cfDNA	30
3	PASEA	Cas9	Isothermal (RPA)	KRAS (PDAC)	Wild-type allele shearing	91.4% pre-chemotherapy, 44.83% post-chemotherapy	Rapid (20 min), clinical correlation	Plasma ctDNA	31
4	Impedimetric biosensor	dCas9	None	PIK3CA exon 9	Label-free graphene-based biosensor	0.65 nM LOD	Label-free, rapid (40 s)	Spiked blood	32
5	MEF-based biosensor	Cas12a	None	BRCA-1 (breast cancer)	Metal-enhanced fluorescence *via* AuNP	0.34 fM LOD	Dual colorimetric/fluorescence detection	Spiked 5% human serum	36
6	DSN-assisted fluorescent biosensor	Cas12a	None	BRCA-1	Duplex-specific nuclease-based signal recycling	365 aM (fluorescence), 1 pM (colorimetric)	Dual-mode, amplification-free detection	Serum	37
7	CRISPR/Cas 12a v/s ddPCR sensitivity comparison	Cas12a	PCR	EGFR L858R, T790M	Fluorophore-quencher (FQ)-based detection	0.005% allele freq. (CRISPR), 0.05% allele freq. (ddPCR)	Improved sensitivity and reduced runtime compared to ddPCR	Plasma	33
8	DETECTR-like platform	Cas12a	Isothermal (RPA)	EGFR (exon 19 del, L858R, T790M)	Fluorophore-quencher integrated into lateral flow system	1 copy per μL	Simultaneous detection of two EGFR mutants on same strip	Plasma cfDNA	34
9	CASMART	Cas12a	Isothermal (RPA)	EGFR L858R	Collateral cleavage of ssDNA-FQ reporter	0.3 copies per μL	Fast (1 h), fluorescence-based digital quantification	Simulated cfDNA samples	35
10	CALSA	Cas12a	None	BRCA-1	Autocatalytic feedback loop	Single-molecule sensitivity	Rapid real-time detection, one-pot reaction	Cell line derived cfDNA	41
11	MOF-based fluorescent biosensor	Cas12a	None	KRAS (lung cancer)	MOF-linked fluorophore on magnetic beads	5.6 fM	10^4-fold signal boost, clinical serum	Serum cfDNA	39
12	DFM-AuNP biosensor	Cas12a	None	BRCA-1	Aggregation-based signal (color shift)	0.081 fM	Dual-color dark field, image quantification	Cell lysate	38
13	RPA + PAM-engineered Cas12a	Cas12a	Isothermal (RPA)	EGFR, KRAS, TP53 (NSCLC)	Artificial PAMs + mismatch-tuned crRNA	100 aM; 0.02% VAF	Multiplex mutation detection	Plasma cfDNA	40
14	HiCASE	Cas13a	PCR	EGFR T790M/C797S	PCR-based CRISPR, coupled with the restriction enzyme	88.1% sensitivity, 100% specificity	Uses only 40 μL of plasma, distinguishes *cis*/*trans* mutants	Plasma cfDNA	42
15	Electrochemical sensor	Cas14	RPA	EGFR L858R (NSCLC)	Electron transfer on gold electrode surface	0.34 fM	Electrochemical signal change proportional to target concentration	Spiked sample	44

ability of Cas14 to cleave ssDNA is distinct because it can cleave ssDNA without a restrictive protospacer adjacent motif (PAM), but, importantly, Cas14 does require T-rich PAM sequences for dsDNA targeting, such as TTTG.[18]

3. Application of CRISPR-Cas system in cfDNA-based cancer diagnosis

3.1. Cas9-based systems

The foundational approach was using CRISPR-Cas9 to deplete the wild-type sequence, capitalizing and employing the site-specific cleavage mechanism of Cas9 cleaving the wild-type EGFR DNA fragment at the sequence site adjacent to a PAM sequence, sparing the EGFR mutant (exon 19 deletion) DNA fragments. After this targeted cleavage, the remaining mutant DNA strands are amplified through PCR, thus enriching the mutant fragments and enhancing detection limits down to 0.01% allele frequency using Sanger sequencing. A post-cleavage PCR showed a 1000-fold improvement over unprocessed samples, establishing it for Cas9-driven signal-enhancement assay to combat the challenge of low target abundance of target cfDNA.[29] Continuing along this line of investigation, Boyeon Kim *et al.* introduced CRISPR-CPPC (CRISPR combined with post-PCR cfDNA), focusing on targeting the T790M resistance mutation. The T790M mutation in the EGFR gene often appears in some patients after the use of first- or second-generation tyrosine kinase inhibitor (TKI) therapies, thus creating resistance. Here, cfDNA-based mutant detection can assist with modifying therapy *via* timely intervention. In the CPPC system, PCR is done before cleavage of wild-type sequences using a Cas9–sgRNA (single guide RNA) complex. This strategy elegantly addresses the issue of high fragmentation that can mislead PCR amplification if pre-amplification cleavage is performed. The sample post-amplification was quantified with ddPCR, achieving a 13-fold signal enhancement and increasing detection sensitivity from 43.8% with ddPCR alone to 93.8% when ddPCR was combined with CRISPR.[30] While CRISPR-CPPC offered significant enrichment, its reliance on a subsequent PCR step and ddPCR for quantification still presents a workflow complexity.

Moreover, to overcome the technical bottleneck of detecting rare KRAS mutant alleles in the liquid biopsy samples with a high-background wild-type cfDNA environment, the Cas9-based programmable enzyme-assisted selective exponential amplification (PASEA) system was introduced. PASEA harnesses CRISPR-Cas9-mediated selective cleavage of wild-type alleles and adds recombinase polymerase amplification (RPA) to enrich plasma KRAS mutants. By providing a continuous digest of the non-mutated alleles during amplification through RPA, PASEA enables preferential enrichment of rare mutants, resulting in sufficient acquisition of the mutant alleles for robust detection of the mutant even when some clinical samples have low ctDNA burden. In a cohort of 153 patients, PASEA showed a sensitivity of 91.4% in pre-chemotherapy patients and was successfully used to track therapeutic response with greater resolution compared to the well-validated protein biomarker CA19-9. PASEA has all the characteristics of an assay designed for the clinical detection lab: isothermal, 20-minute turnaround time, and no need for expensive thermocyclers.[31] In addition to amplification-dependent strategies, catalytically inactive Cas9 (dCas9) has been applied in label-free electrochemical biosensing platforms. An example of this is a system developed by Zihni Onur Uygun *et al.* where those systems utilized dCas9-sgRNA complexes immobilized to graphene oxide screen-printed electrodes to detect PIK3CA exon 9 mutations electrochemically and based on impedance. They performed this detection without labeling any moieties, amplifying them, or cleaving them, thus providing a simple-to-perform platform that allowed for rapid detection of the target in 40 seconds, high recovery (>96%) when spikes with blood samples were used, and a limit of detection of 0.65 nM.[32] This work is a good example of utilizing Cas9 for rapid amplification-free detection and point-of-care diagnostics.

3.2. Cas12a-based systems

To enhance the sensitivity of EGFR T790M mutant detection, newer approaches switched to Cas12a-based detection systems as they have high collateral cleavage activity. One assay multiplexed PCR with the Cas12a enzyme to target two mutants of EGFR: T790M and L858R. The assays utilized a fluorophore-quencher reporter system that was cleaved by Cas12a collateral activity, and the fluorophore signal was used as the signal for detection. This assay provided a limit of detection (LOD) of 0.005%, which was an improvement compared with ddPCR (LOD = 0.05%). Also, the total time for the proposed was <3 hours compared to over 5 hours for ddPCR. Importantly, the assay was able to detect the T790M resistance mutation in patient plasma when ddPCR failed, showing Cas12a's enhanced sensitivity for low-frequency variants.[33] Additionally, another strategy modeled on DETECTR was made of a lateral flow device that was based on Cas12a, which combined isothermal amplification (RPA) and portable readouts. With attomolar-level sensitivity, this assay was able to detect two EGFR mutations simultaneously: exon 19 deletion with T790M and L858R with T790M on the same strip. The entire assay was conducted at 37 °C, meaning costly equipment was unnecessary and the assay format was portable for field diagnostics (Fig. 2).[34] The next step of advancement came with CASMART, a one-step isothermal Cas12a-based platform that integrated recombinase polymerase amplification (RPA) and digital microfluidics. It depicted ultra-low detection limits (0.3 copies per μL) and highly accurate allele quantification of EGFR L858R mutant, circumventing the need for thermocycling and thus reducing assay time by half compared to dPCR. This digital system is a specifically promising advancement for use in resource-limited clinical settings, leveraging convenient result readouts.[35]

For the detection of BRCA1 mutations, a significant genetic alteration associated with breast cancer, several amplification-free Cas12a systems have emerged, crucial for predicting hereditary cancer risk and personalizing treatment strategies. One study introduced a metal-enhanced fluorescence (MEF)

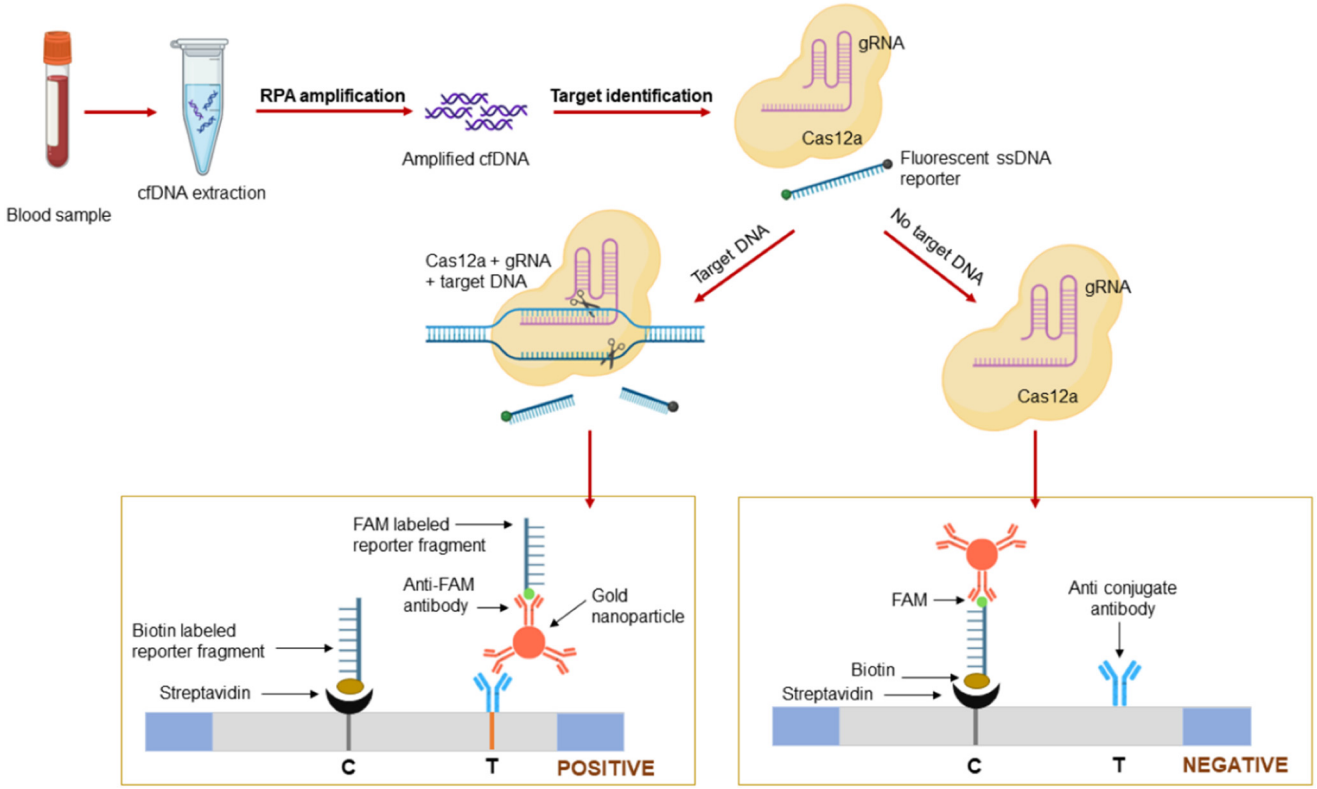

Fig. 2 DETECTR-like method – a schematic depicting the workflow and detection mechanism of CRISPR/Cas12a-based lateral flow detection of mutant cfDNA fragments. The ssDNA reporter was labeled with FAM and biotin at the 5′ terminus and 3′ terminus, respectively. The lateral flow strip used AuNP anti-FAM antibody to produce the detection signal. The test (T) band on the lateral flow strip appeared only when the ssDNA-labeled-reporter was cleaved by CRISPR/Cas12a collateral cleavage, triggered by EGFR mutant identification.

strategy by conjugating fluorophore-linked ssDNA to gold nanoparticles (AuNPs). Upon recognition of BRCA1 cfDNA, Cas12a's *trans*-cleavage activity disrupted the MEF effect, triggering both a fluorescence shift and a visible color change in the solution. The key highlight was that this platform achieved detection within 30 minutes and a LOD of 0.34 fM, and offered a dual optical readout, supporting point-of-care (POC) potential through visual validation and sensitivity enhancement without amplification.[36] Further advancing signal output without compromising simplicity, another study targeting BRCA1 developed a dual-mode detection system integrating Cas12a cleavage with duplex-specific nuclease (DSN)-mediated signal recycling. This platform used cascade processes in which fluorescent signals were amplified through DSN in addition to destabilizing AuNPs to induce a colorimetric response. They generated a fluorescence-based LOD of 365 aM and a colorimetric LOD of 1 pM. This amplification-free assay advances the analytical capabilities of detecting the target ctDNA through the identification of both fluorescence-based and colorimetric signal types.[37] Another study targeting BRCA1 cfDNA detection constituted of Cas12a coupled with single nanoparticle dark-field microscopy (DFM) for label-free, amplification-free cfDNA detection. The group developed a visual readout using the aggregate state of AuNPs, imaged using DFM, visually scanned in color, and subsequently performed quantitative analysis using machine learning algorithms. The assay produced a LOD of 0.081 fM in 40 min which eliminated the need for both enzymatic signal enhancement and amplification procedures and therefore allowed for image-based quantification, which eventually incorporates automation into high-throughput diagnostics.[38] These BRCA1-focused platforms are groundbreaking with similar momentum by reducing amplification dependence while increasing user ease, which is crucial for POC diagnostics. Furthermore, Cas12a was combined with a metal–organic framework (MOF)-based fluorescence amplification platform to detect rare KRAS mutations at codon 12 in pancreatic ductal adenocarcinomas (PDAC) and cohorts of lung and colorectal malignancies. Amino-functionalized MOFs worked as highly capacitated carriers of fluorophores, capped off with ssDNA linkers that were cleaved upon target detection. Activation of Cas12a by KRAS mutant ctDNA led to linker cleavage, fluorophore release, and a sharp fluorescence rise. Magnetic separation aided in achieving background-free measurements and offered a LOD of 5.6 fM in spiked serum samples.[39] Unlike Cas9-based PASEA which relies upon enzymatic amplification, the MOF-Cas12a approach increased signal intensity *via* nanomaterial-based payload design, thus providing a high-performance biosensing that does not utilize enzymatic amplification. Besides the detection of one mutation at a time, the critical requirement for multiplexed ctDNA mutation detection in NSCLC was addressed by utilizing a CRISPR RNA (crRNA) library that targeted EGFR,

KRAS, and TP53 simultaneously. To bypass the PAM sequence limitation, artificial PAM site sequences were incorporated during amplification, and to improve the base mismatch discrimination, engineered crRNAs incorporated intentional base mismatches. This system achieved the detection of mutations down to 0.02% variant allele frequency in just 50 minutes. Validated in patient-derived plasma, the platform promises broad-spectrum mutation screening for both early detection and resistance monitoring. This multiplex design is highly convenient for detecting multiple targets, which would reflect the mutational landscape in heterogeneous tumors.[40]

Finally, one of the most forward-looking adaptations is CALSA (CRISPR-Cas autocatalysis amplification driven by LNA-modified split activators), which provides a new mechanism for signal amplification that does not require external nucleic acid preamplification. CALSA is built on the catalytic activity of LbCas12a but in a fundamentally different design with split single-stranded (ssDNA) DNA activators that activate Cas12a upon assembling in the presence of a target sequence. The key difference is that activation of Cas12a does not only induce the cleavage of a reporter molecule but also self-sustains a positive feedback loop where each cleavage generates new activators that maintain the reaction. To further optimize the system, the split activators also contained locked nucleic acid (LNA) modifications that enhanced hybridization fidelity and directed Cas12a's *trans*-cleavage activity to specific sequences. This site-directed cleavage minimizes off-target effects and boosts both efficiency and specificity. As a result, CALSA achieves rapid, one-pot, isothermal detection of genomic and cell-free DNA (cfDNA), with the ability to discriminate even single-nucleotide mismatches, all under physiological temperature conditions. This effect not only shortens assay workflows but also accelerates signal turnover. This platform was validated over several tumor-derived cfDNA and genomic DNA model samples, confirming its broad diagnostic potential.[41]

3.3. Cas13a-based systems

Expanding the enzyme toolbox, HiCASE implemented Cas13a in combination with PCR and restriction enzyme digestion to target and specifically eliminate wild-type sequences. Cas13a has the uniqueness of targeting RNA instead of DNA unlike Cas9 or Cas12a, and is transformed into a detection assay by converting the PCR products to RNA before detection. One huge finding was that HiCASE was able to detect down to 0.01% variant allele frequency and could differentiate between the *cis*- and *trans*-configured T790M/C797S mutations, which will be important for implementing personalized therapy decisions. HiCASE also compared its ability *versus* ddPCR and super-ARMS, concluding that Cas13a combined with PCR produced a high level of sensitivity and improved positional specificity in distinguishing between the *cis* and *trans* mutants. However, Cas13a targets the RNA, which limits its applicability to DNA-based targets, as assays with Cas13a require a prior step of converting DNA fragments into RNA to be targeted by Cas13a, which adds additional steps, time, and enzymes, thus increasing the complexity of the assay and reducing its broader applicability.[42] Overall, these studies may present many various and astounding advancements—from the early Cas9 enrichment method to the advanced systems using Cas12a and Cas13a technologies—delivering digital quantification, positional mutation characterization, and lateral flow-based deployment. These technical advancements reflect an ongoing trend to surpass amplification-dependent strategies while simultaneously improving sensitivity, portability, and usability for POC diagnostics.

3.4. Cas14a-based systems

Cas14a is yet another Cas enzyme which is ultra-small in size, bestowing the ability to discriminate between the target sequences. Moreover, it actively cleaves the both double-stranded DNA (dsDNA) and single-stranded DNA (ssDNA), coupled with the collateral ssDNA cleavage activity similar to Cas12a and Cas13a.[43] However, Cas14a is not extensively investigated for the applicability in cfDNA-based cancer diagnostics. The first study reporting the applicability of Cas14a in cfDNA diagnostics is by Qi *et al.*,[44] incorporated into a electrochemical biosensor. It was applied for the detection of the EGFR L858R mutant in NSCLC cases. The assay coupled the isothermal amplification of target cfDNA *via* the primer exchange reaction (PER); in turn it activates the CRISPR-Cas14a system. The activated system releases substrate ssDNA-MB, subsequently leading to the transfer of electrons which generates a fluctuated electrochemical redox signal on the surface of gold electrode. In contrast, in the absence of target EGFR L858R mutant, the electrochemical signal does not encounter any change. This platform promises a reproducible, specific and significant recovery rate, with high potential in mutant detection.[44]

The distinctive collateral cleavage characteristic of Cas14a in a PAM-independent manner widens its scope for multiple gRNA target creation and its applicability for multiple targets in fields other than cancer diagnostics. A study has reported Cas14-DETECTR, coupling Cas14 with RPA for detection of single nucleotide polymorphisms (SNPs). This study utilized a phosphorothioate (PT)-labelled 5′ primer which is cleaved by Cas14 upon target recognition, thus producing the fluorescent signal.[45] Perhaps, the key features of Cas14 can be explored further in cfDNA detection to improve cancer diagnostics.

4. Translational outlook and future perspectives

The utilization of CRISPR/Cas systems for circulating cell-free DNA (cfDNA) diagnostics represents a significant development in molecular oncology. While other technologies, including ddPCR and NGS, are limited by lengthy processing times, high equipment requirements, and limited sensitivity for variants in early-stage oncology or low frequency, CRISPR-based platforms present a more attractive option. The value of a CRISPR-based

solution becomes increasingly evident in more recent studies, which show a clear trajectory of development from single-target, amplification-based assays to multiplexed, amplification-free, and signal-enhancement biosensors, all aimed at improving the clinical utility of cfDNA-based diagnostics.

One of the most revolutionary trends is the purposeful and deliberate shift away from nucleic acid amplification. Amplification-free systems, particularly those that are intended for BRCA1 cfDNA detection, illustrate how the collateral cleavage activity of Cas12a can be combined with signal transduction strategies based on nanomaterials to reduce assay complexity and provide additional application opportunities in low-resource settings. In addition, several systems have introduced dual-mode detection systems that incorporate fluorescence and visible colorimetric output to improve analytical robustness and support usability. Finally, time-to-result is another significant area where CRISPR assays assertively outperform legacy methods, and while many CRISPR-affiliated platforms report runtimes between 30 and 60 minutes, ddPCR or NGS can take, in some cases, days to yield results. Some CRISPR-based systems have even shown better sensitivity compared to regular molecular platforms, allowing detection of mutant allele frequencies as low as 0.005%. The CALSA platform represents a new era of CRISPR diagnostics by harnessing sustained, autocatalytic, LNA-modified split activators. One-pot, isothermal cfDNA detection without preamplification has simply allowed rapid detection of cfDNA. Further, such platforms would be highly applicable for clinical utility combined with digital detection modes (*e.g.*, dark-field microscope or smartphone), leveraging machine learning algorithms for result interpretation, resulting in user-friendly diagnostic platforms. In addition to speed and sensitivity, the operational simplicity of these assays makes them a plausible option for acute diagnosis or long-term monitoring of patients during treatment. This equates to earlier detection of relapse for timely intervention and more accurate modifications to treatment, ultimately leading to better patient outcomes.

Reinforcing these academic innovations is a growing body of intellectual property that points toward commercial readiness. Two key patents exemplify this shift. WO/2019/071051 (ref. 46) describes a CRISPR-based lateral flow assay that includes effector proteins, including Cas12 or Cas13, sequence-specified guide RNAs, and reporter constructs to allow multiplexed, amplification-free nucleic acid detection. The platform utilizes biotin- or fluorophore-labeled probes that are captured on a multi-band test strip that features portable, visually interpretable readouts. This device would particularly be well-fitted for rapid cfDNA-based diagnostics based on mutation profiling as the developed assay obviates the need for expensive thermocyclers. Another patent, WO/2021/046257,[47] describes a multiplexed CRISPR-Cas13 diagnostic system targeting cancer RNA biomarkers that utilizes reverse transcription recombinase polymerase amplification (RT RPA), T7 transcription, and two Cas13 effectors (LwaCas13a and CcaCas13b) to target distinct RNA signatures. The assay, including FAM and Alexa 488, allows for the identification of differential RNA splicing variants or isoforms, which is paramount for cancer. This system can utilize both fluorescence- and lateral flow-based readouts, thus accommodating centralized laboratory and POC assays. Collectively, these patents serve as a technological and regulatory path that informs the viability of CRISPR diagnostics going from bench to bedside.

While these advancements have been made, there remain several barriers preventing the widespread acceptance of these assays in clinical practice. Existing CRISPR-based platforms rely on multi-step workflows that include nucleic acid extraction, amplification, and manual result interpretation, introducing risks of contamination, variability, and longer assay times. Variability is unavoidably introduced in the assays due to the lack of standardized protocols in at least three aspects, crRNA design, enzyme optimization, and detection methods, which complicate assay reproducibility. Importantly, regulatory uncertainty, insufficient large-scale clinical validation, and manufacturing challenges including scalability and reagent stability stand as the major constraints. Henceforth, associated regulatory constraints on the CRISPR system are impacting the clinical translation of these assays to *in vitro* diagnostics (IVD) from FDA. Further, to scale up CRISPR component production to meet good manufacturing product (GMP) requirements, there is a need for improvements in consistency, stability, and cost. However, to step up the clinical utility of the CRISPR-based diagnostic system, there is a crucial requirement to design large-scale clinical trials comparing the performance of CRISPR assays to the current gold-standard methods NGS or ddPCR. Cross-center trials with sufficient statistical significance are critical to providing health-related decisions based on the clinical utility and effectiveness of the CRISPR-Cas-based assays to momentously advance cfDNA from an exploratory research biomarker to an integrated component in measures of precision oncology. Moving ahead, the development of these platforms demands the interdisciplinary collaboration of molecular biologists, engineers, and clinicians. The advent of lyophilized assay kits, AI-driven crRNA libraries, and scalable microfluidic technology will even more rapidly accelerate the translational pipeline to deliver accurate, accessible, and actionable information to patients everywhere.

5. Cost comparison of CRISPR-based diagnostics against conventional methods

The four primary factors that critically govern the cost expenditure of molecular diagnostic assays are cost of assay reagents and consumables (master mixes, primers/probes, enzymes), instrumentation (capital and maintenance for equipment like thermocyclers and sequencers), skilled personnel, and infrastructure/overhead expenses. Key determinants of per-sample cost include test volume, which dilutes fixed expenses across a greater number of assays, and assay complexity, as multifaceted workflows, such as next-generation sequencing (NGS), inherently entail higher

Table 2 Comparative cost analysis of CRISPR-based assays against conventional methods

Cost components	CRISPR/Cas assays	Quantitative PCR	Digital droplet PCR	Next-generation sequencing
Assay reagents	1. CRISPR/Cas components: ~$150 (0.1 mg)[a] 2. sgRNA: $114, $295[b] 3. Lateral flow strips, device fabrication material, nanomaterials	1. qPCR Master Mix: ~$857 (200 reactions)[c] to ~$1064 (200 reactions)[d] 2. Primers 3. Taqman probes	1. ddPCR Master Mix: ~$232 (200 reactions)[e] 2. ddPCR droplet generation oil: ~$335 (7 × 10 ml)[f] 3. Primers 4. Fluorophore-tagged probes	Materials cost (MiSeq™ and HiSeq™): ~$1407–$6385 (ref. 49)
Equipment cost (capital)	1. Simple heat block for isothermal assay–low cost 2. Minimal instrument for signal readout	~$8000 or above for real-time thermocyclers	More expensive than qPCR, as it includes a thermocycler, a droplet generator with cartridges, and droplet readers	Materials cost (MiSeq™ and HiSeq™): ~$113–$310 k (ref. 49)
Infrastructure	Suitable for low-resource settings	Require a laboratory setup for instrument operation and result visualization on computer system	Massive instrumentation	Massive instrumentation and IT support
Trained personnel	Basic molecular skills for simpler protocols; minimal training for point-of-care device based testing	Standard molecular biologist; thermocycler operator	Trained operator required for optimizing and operating droplet generator and data analysis	Highly trained for library preparation + bioinformatics tools utilization for data analysis

[a] GenCRISPR™ NLS-wtSpCas9, his-tag (GenScript). [b] Pre-synthesized crRNA from IDT (2 nmol from standard desalt and HPLC purification, respectively). [c] TaqMan™ Universal PCR Master Mix. [d] Verso 1-step RT-qPCR Kit, SYBR Green, ROX. [e] ddPCR Supermix for probes (no dUTP). [f] Droplet generation oil for probes.

equipment and reagent outlays. Continuous advancements in automation and reagent innovation consistently work to lower these costs by streamlining workflows and reducing manual labor (Table 2).

Traditional molecular diagnostic platforms are dependable; however, they are often found to be expensive. For example, although a quantitative polymerase chain reaction (qPCR) carries a moderate price due to the infrastructure requirements and associated instrumentation, ddPCR carries a cost that includes a probably steeper price as it achieves a higher level of accuracy using droplet distribution of samples with digital computation to determine the absolute quantification of copy numbers. One study previously observed that the cost of ddPCR reagents was €2.80 per reaction compared to the cost of reagents for qPCR, which was between €1.20 and €1.60.[48] Next-generation sequencing (NGS), designed to provide unmatched breadth in genomics, has the highest cost per sample. That is largely driven by instrumentation costs, complex library preparation and bioinformatic analysis.[49] The utility of these technologies relies on the volume of testing, the complexity of assays, and clinical need.

However, cost-effective assays can be developed with increased accessibility when the exact identification of a target is important, without it being necessary to quantify precisely or produce new mutants for an unknown. This is applicable to point-of-care testing (POC), especially in terms of precision oncology when a patient's disease progression and treatment responses need to be regularly monitored to predict relapse as early as possible. The vision for economically affordable cancer diagnostic testing using CRISPR/Cas approaches in POC applications is perhaps transformative due to benefits including single-base detection precision, versatility for isothermal methods and amplification-free use, and electrochemical sensing. Further reduction in cost is certain with the integration of automation combined with isothermal or amplification-free approaches.

One important coupled cost in the cell-free DNA (cfDNA)-based cancer detection space is the cost of cfDNA extraction kits. Most cfDNA extraction kits range in cost from $610 (MagMAX™ Cell-Free DNA Isolation kit) to $1624 (QIAamp ccfDNA/RNA kit) for 50 preps, translating to $20–$32 per prep, with other kits generally falling under this range. This cost can be seen as an initial cost barrier for cfDNA-based diagnostic platforms. However, acknowledging the critical importance of minimally invasive cfDNA-based diagnosis in combating cancer, future improvements in cfDNA enrichment without costly kits are vital. Furthermore, fabricating assays into lateral flow or paper strips can enhance portability, require smaller test sample volumes, and reduce reagent consumption per test, thereby cutting down the overall per-test cost. Point-of-care platforms, such as miSHERLOCK, have demonstrated saliva-based SARS-CoV-2 detection at approximately $15 per test, a cost reducible to $11 through the reuse of simple electronics and heaters.[50] Similarly, paper-strip CRISPR assays can cost under $5 when fabricated with inexpensive substrates. The major assay expense (around $9) in some instances has been attributed to the use of commercial enzymes like RPA and reverse transcriptase, which is expected to decrease with scale.[51] Various groups are actively developing inexpensive CRISPR-Dx POCs; for example, a LAMP/CRISPR HPV assay utilizing a gold leaf electrode achieved a sensitivity of 10^4 copies per test at a cost of approximately $2.30 per test.[52] Importantly, some studies shown that CRISPR paired with nucleic acid detection procedures can eliminate the need for pre-amplification without compromising sensitivity, further

reducing reagent and equipment costs and obviating the need for highly trained personnel.

Importantly, many CRISPR readouts are colorimetric/lateral flow-based and thus have a visual interpretation or some readers are available for very inexpensive uses that eliminate the need for expensive fluorescence imagers and sequencers. While cfDNA extraction has a similar base cost for every method, CRISPR platforms will save substantially on equipment costs and should also eliminate the need for having thermocyclers which would reduce all the overhead infrastructure costs including training. In addition to these cost savings, when planning a POCT, cost considerations underlie every step of the workflow, from collecting and preparing the sample to the layer of biomolecular signal readout (whether using expensive colorimetric reagents or lateral flow readers) including costs for assay reagents and materials, for disposable cartridges and portable readers. Implementing CRISPR-PCT assays into microfluidic devices, if the devices are made from low-cost materials and low-cost manufacturing protocols, should generate affordable overall assay protocols. Microfluidic/electrochemical paper-based analytical devices also have many attractive properties, not least of which is low-cost materials that are portable and disposable, although the availability of high-quality papers may not apply in very low-cost resource settings.

6. Broader applicability of CRISPR/Cas systems

6.1. CRISPR/Cas in viral pathogen detection

The rapid demonstrability of this incredible transferability has already been shown toward significant global health challenges. One of the most prominent is the rapid development and deployment of CRISPR-based assays leveraged against viral pathogens. SARS-CoV-2 exemplified this opportunity. For example, SHERLOCK- and DETECTR-based platforms were rapidly adapted to detection of SARS-CoV-2 nucleic acids, authorized under Emergency Use Authorization (EUA) by the FDA.[18] These platforms typically utilize RNA-targeting Cas13 enzymes in the case of SHERLOCK or DNA-targeting Cas12 enzymes in the case of DETECTR, which themselves used the RNA-guided *trans*-endonuclease activity of Cas13 (in the case of RNA viruses) or triggered nonspecific ssDNA cleavage by Cas12 (in the case of DNA viruses and reverse-transcribed RNA viruses), followed by isothermal amplification, to generate a detectable end-product and an indicator of the presence of viral genomes. For example, Cas13 has been shown to be able to distinguish single-nucleotide polymorphisms (SNPs) in SARS-CoV-2 variants,[53] and amplification-free Cas13-based detection has been demonstrated for SARS-CoV-2 and can detect as little as 100 copies of viral RNA per microliter of nasal swab RNA.[54] The ENHANCE system, with Cas12a, has also been used to detect various nucleic acids from viruses such as HIV, HCV, and SARS-CoV-2.[55] These demonstrations illustrated that the innovative CRISPR framework created for cancer detection could serve as a suitable starting point with only minor modifications to suit diagnostic applications for infectious diseases, genetic conditions, and other disease states where nucleic acid biomarkers are central.

6.2. CRISPR/Cas in therapeutics

CRISPR/Cas systems (specifically, CRISPR/Cas9 systems) are increasingly exhibiting their capabilities not only in diagnostic applications but also as revolutionary platforms for therapeutic uses; in the near future, it may be feasible to precisely alter deleterious gene mutations or interfere with disease-causing genes with an efficiency previously thought impossible. Available gene editing technology is extremely powerful and provides opportunities for treatment of both genetic and acquired diseases at a molecular level. Such applications hold promise for treating a wide variety of disorders, especially cancers, cardiovascular diseases, sickle cell anemia, and neurodegenerative diseases.[56]

Some recent developments have moved so quickly that initial data are translated into successful clinical trials. For example, a gene therapy using CRISPR/Cas9 was implemented for treating β-thalassemia. Therapy was used to repress the expression of BCL11A, which was the repressor, and consequently restored the production of γ-hemoglobin and fetal hemoglobin.[57] Similarly, EDIT-101, created as a genome-edited therapy for Leber congenital amaurosis type 10 (LCA10), used an AAV5 virus vector to deliver the Cas9 system to photoreceptor cells, correcting a mutant intron, and appeared to have therapeutic effects.[58] Despite the ongoing challenges regarding effective and safe *in vivo* delivery, the ongoing development of new delivery vectors and optimized Cas9 variants demonstrates the incredible therapeutic potential of CRISPR technology and its place in the human fight against disease, alongside its diagnostic application.

7. Conclusions

The development of CRISPR-Cas systems has revolutionized cfDNA-based cancer diagnostics by providing the tools to detect low-abundance, fragmented tumor yielding highly specific, programmable, and modular tools, all the while alleviating the constraints of older methodologies such as ddPCR and NGS. Collectively, recent studies reveal a clear move away from amplifying methods to amplification-free, miniaturized platforms, using collateral cleavage, signaling based on nanomaterials, and digital readouts, to achieve rapid, sensitive, and multiplexed detection which have mainstreamed these platforms into clinical applications. These innovative methods of cfDNA detection will be particularly beneficial for early detection, monitoring of therapy, and in settings with limited resources. As appropriate regulatory oversight and standardized reporting of results emerge, coupled with a clearer understanding of the commercialization of CRISPR systems, including their scale, patents, and market interest, a pathway to scalable, point-of-care applications of CRISPR diagnostics will

become more evident. With further advancements, the liquid biopsy-based detection of cfDNA could be a critical part of precision oncology.

Conflicts of interest

There are no conflicts to declare.

Abbreviations

CRISPR	Clustered regularly interspaced short palindromic repeats
Cas	CRISPR-associated proteins
cfDNA	Cell-free DNA
ddPCR	Digital droplet polymerase chain reaction
NGS	Next-generation sequencing
LB	Liquid biopsy
CTCs	Circulating tumor cells
EVs	Extracellular vesicles
ctDNA	Circulating tumor DNA
EGFR	Epidermal growth factor receptor
KRAS	Kirsten rat sarcoma virus
BRCA-1	Breast cancer gene 1
SEPT9	Septin-9
NSCLC	Non-small-cell lung cancer
PDAC	Pancreatic ductal adenocarcinoma
FDA	Food and Drug Administration
EMA	European Medicines Agency
POC	Point-of-care
sgRNA	Single guide RNA
crRNA	CRISPR RNA
tracrRNA	Trans-activating crRNA
dCas9	Dead Cas9
PAM	Protospacer adjacent motif
dsDNA	Double-stranded DNA
ssDNA	Single-stranded DNA
DETECTR	DNA endonuclease targeted CRISPR trans reporter
HPV	Human papillomavirus
RPA	Recombinase polymerase amplification
ssRNA	Single-stranded RNA
SHERLOCK	Specific high-sensitivity enzymatic reporter unlocking
CPPC	CRISPR combined with post-PCR cfDNA
TKI	Tyrosine kinase inhibitor
PCR	Polymerase chain reaction
PASEA	Cas9-based programmable enzyme-assisted selective exponential amplification
CA19-9	Carbohydrate antigen 19-9
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
CASMART	CRISPR associated mutation allele rapid test
MEF	Metal-enhanced fluorescence
AuNPs	Gold nanoparticles
LOD	Limit of detection
DSN	Duplex-specific nuclease
DFM	Dark-field microscopy
MOF	Metal–organic framework
CALSA	CRISPR-Cas autocatalysis amplification driven by LNA-modified split activators
LNA	Locked nucleic acid
HiCASE	High-sensitivity PCR-Cas13a with specific restriction enzyme detection
ARMS PCR	Amplification refractory mutation system polymerase chain reaction
RT RPA	Reverse transcription recombinase polymerase amplification

Data availability

The present article submission is a review and there are no experimental data available for the same from the authors.

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