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Review article

HIV infection detection using CRISPR/Cas systems: Present and future prospects

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ABSTRACT

Human immunodeficiency virus (HIV) infection poses substantial medical risks to global public health. An essential strategy to combat the HIV epidemic is timely and effective virus testing. CRISPR-based assays combine the highly compatible CRISPR system with different elements, yielding portability, digitization capabilities, low economic burden and low operational thresholds. The application of CRISPR-based assays has demonstrated rapid, accurate, and accessible means of pathogen testing, suggesting great potential as point-of-care (POC) assays. This review outlines the different types of CRISPR/Cas systems based on Cas proteins and their applications for the detection of HIV. Additionally, we also offer an overview of future perspectives on CRISPR-based methods for HIV detection, including advances in nucleic acid amplification-free testing, improved personal testing, and refined testing for HIV genotypes and drug-resistant strains.

1. Introduction

Human immunodeficiency virus (HIV) causes acquired immune deficiency syndrome (AIDS), which has resulted in approximately 650,000 deaths globally in 2021 [1]. Effective HIV testing is essential for the timely detection of infected persons that allows for control of HIV spreading in the population, rapid initiation of treatment for HIV-infected people, and induction of stable/undetectable disease, which serves a critical role in the whole spectrum of HIV prevention and control.

Currently, the mainstream methods for detecting HIV nucleic acids are polymerase chain reaction (PCR)-based, such as real-time fluorescent quantitative PCR and fluorescent probe PCR, which can yield satisfactory accuracy and specificity [2]. Other existing methods, containing enzyme-linked immunosorbent assay (ELISA), quantitative viral growth assay (QVOA), tat/rev induced limiting dilution assay (TILDA), and flow cytometry-fluorescence in situ hybridization (Flow-FISH), are also used to a greater or lesser extent for HIV detection [3–6]. However, performing a complete and high-quality PCR (or ELISA, QVOA, TILDA, Flow-FISH, etc.) requires trained personnel, high-value equipment, and a long-time horizon [7,8]. These factors limit the accessibility of PCR and other highly professional methods in remote areas, making it challenging to use as an efficient POC test for HIV detection.

The limitations of these methods for HIV detection in resource-limited settings and POC environments have motivated the exploration for alternative detection methods such as the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-related (Cas) system-based assays. CRISPR/Cas system is a naturally occurring adaptive immune system found in a wide range of bacteria and archaea, functioning to defend against invasion by phages, plasmids, and other exogenous genetic elements [9]. The classical working mechanism of the CRISPR/Cas system is that guided by the sequence-diverse CRISPR RNA (crRNA), the complex identifies secondary invading exogenous DNA or its transcript with the protospacer adjacent motif (PAM) limit, and induces DNA or RNA damage through the nucleic acid endonuclease activity of the Cas protein, thereby blocking the infection [10]. Due to its programmability, ease of operation, and the unique advantages of different Cas proteins, the assays based on the CRISPR/Cas system have proven promising as the next generation of molecular diagnostics [11,12]. It is important to note that different types of Cas proteins or even the same type from different bacterial species may have varying requirements for PAM sequence lengths and base ratios. Therefore, when

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considering the target gene and its application, it is necessary to choose an appropriate Cas protein from a species that possesses a compatible PAM sequence.

Ever since the discovery of the CRISPR/Cas system's ability to edit genes, it has garnered significant enthusiasm from researchers [13,14]. Hitherto, applications derived from this system for gene regulation, live cell imaging, and pathogen detection has also received considerable attention. In recent years, with the spread of the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), an increasing number of assays have been developed using the CRISPR/Cas system and related summaries were following [15,16]. However, as far as we know, there is a lack of systematic reviews concentrating on HIV testing regarding these methods. This review begins with an introduction to each type of CRISPR/Cas system based on signature protein. Subsequently, we provide a description of reported CRISPR/Cas-based assays for HIV, highlighting their principles, and finally, offering our own insights into future developments.

2. Brief knowledge of CRISPR/Cas systems based on Cas proteins

The CRISPR/Cas systems can be categorized into two classes, each consisting of three types, with a distinctive endonuclease serving as the signature protein for each type. Comprehensive knowledge of the structural characteristics of these proteins can assist in the formulation of structure-based methods (Table 1).

2.1. Cas9

Cas9 proteins, identified in type II CRISPR systems, are a series of single-effector, multi-structural domain endonucleases that differ greatly from each other in sequence and size [17]. Of these, the most characterized is the Cas9 from *Streptococcus pyogenes* (SpCas9 PDB code: 4OO8), as it was first used for gene editing [13,18,19].

The structure of SpCas9 can be briefly recognized as a distribution of two lobes containing three domains respectively (Fig. 1A). The recognition (REC) lobe consists of the Bridge helix, the REC1 domain, and the REC2 domain. Since SpCas9 is an RNA-directed DNA nuclease, it performs the function by forming an RNA-protein (RNP) complex with a single guide RNA (sgRNA), which is fused from a crRNA and a *trans*-activating crRNA (tracrRNA) that binds to the crRNA in complementary base pairing [13]. And the REC2 domain of the REC lobe is critical for the formation of the Cas9-sgRNA complex while the REC1 domain is responsible for sgRNA: DNA recognition () [18,19]. The nuclease (NUC) lobe is divided into the HNH domain, the RuvC domain, and the PAM-interacting (PI) domain [18,19]. Following recognition of the PAM sequence of the substrate double-stranded DNA (dsDNA) by the PI domain, successful matching of the sgRNA to the target strand induces significant conformational changes in Cas9, keys among which are the migration and activation of the HNH and RuvC domains [18,19]. Catalytically activated HNH and RuvC domains cleave the target and non-target strands respectively, activating homology-directed recombination (HDR) or non-homologous end joining (NHEJ) for gene editing by creating a double-strand break (DSB) [14,20].

Parsing of SpCas9's structure-based working mechanism has inspired its engineering adaptation. Catalytically inactive SpCas9 enzyme, also known as dead Cas9 (dCas9), is one of the most common components of pathogen detection tools based on the CRISPR/Cas system. Amino acid mutations D10A and H840A introduced in the RuvC and HNH domains, respectively, cause dCas9 to lose its DNA cleavage activity but maintain its DNA recognition and binding properties [21,22]. By leveraging CRISPR/dCas9 system's programmable RNA-directed DNA targeting capabilities and fusing or coupling other elements (e.g., the quantum dot (QD)), the detection of multiple pathogens and amplification of their detection signals can be achieved [23]. By only introducing H840A in the HNH domain or D10A in the RuvC domain, a SpCas9 variant called Cas9 nickase (Cas9n) is obtained. Depending on the design of the sgRNA, Cas9n targets any strand of the DNA for precise cleavage and formation of a single-strand nick (SSN) [24]. Using 2 sgRNAs directed to opposite DNA strands, Cas9n can facilitate paired nickase modification, a DSB with a significantly fewer off-target mutagenesis than that caused by the original Cas9 [25]. Due to the programmable nature of the sgRNA, Cas9n can easily create the DNA nick with high flexibility, which provides a multi-purpose alternative to isothermal amplification reactions based on a cyclic nicking-extension-displacement scheme, thus offering great potential for efficient detection of pathogens.

Notably, in vitro experiments have demonstrated that SpCas9 is a single-turnover enzyme, meaning that SpCas9 binds stably to cleavage products, whereas Cas9 from *Staphylococcus aureus* (SauCas9) is a multiple-turnover enzyme [26]. Although SauCas9 requires a more restrictive PAM sequence than SpCas9, the multi-turnover nature of SauCas9 to rapidly cleave substrate DNA may allow it the potential to present a stronger signal when detecting pathogens, which has not yet been further reported [26,27].

Table 1
General characterizations of different Cas proteins.

Cas protein	Type	Target	Guider	*Trans*-activity	Nuclease domain	Additional messages
Cas9	II	dsDNA	sgRNA	-	HNH, RuvC	Multiple purposed variants have been developed
Cas12	V	dsDNA	crRNA	+	RuvC	High specificity; widely used for pathogen detection
Cas13	VI	RNA	crRNA	+	HEPNs	No PAM restrictions
Cas14	V	dsDNA, ssDNA	sgRNA	+	RuvC	Compact structure; high specificity; substrate selection by PAM; not currently used in HIV testing
Cas3	I	dsDNA	crRNA	-	HD	Large fragment destruction of DNA; ill-suited for precise gene editing; not currently used in HIV testing
Cas10	III	RNA	crRNA	+	HD	Also possesses adenylate cyclase and DNA polymerase activity; not currently used in HIV testing
Csf1	IV	Unknown	pseudo-A RNA	-	Unknown	Remaining Mysterious; not currently used in HIV testing

2.2. Cas12

Cas 12 is a type V Cas nuclease that comprises two highly valued

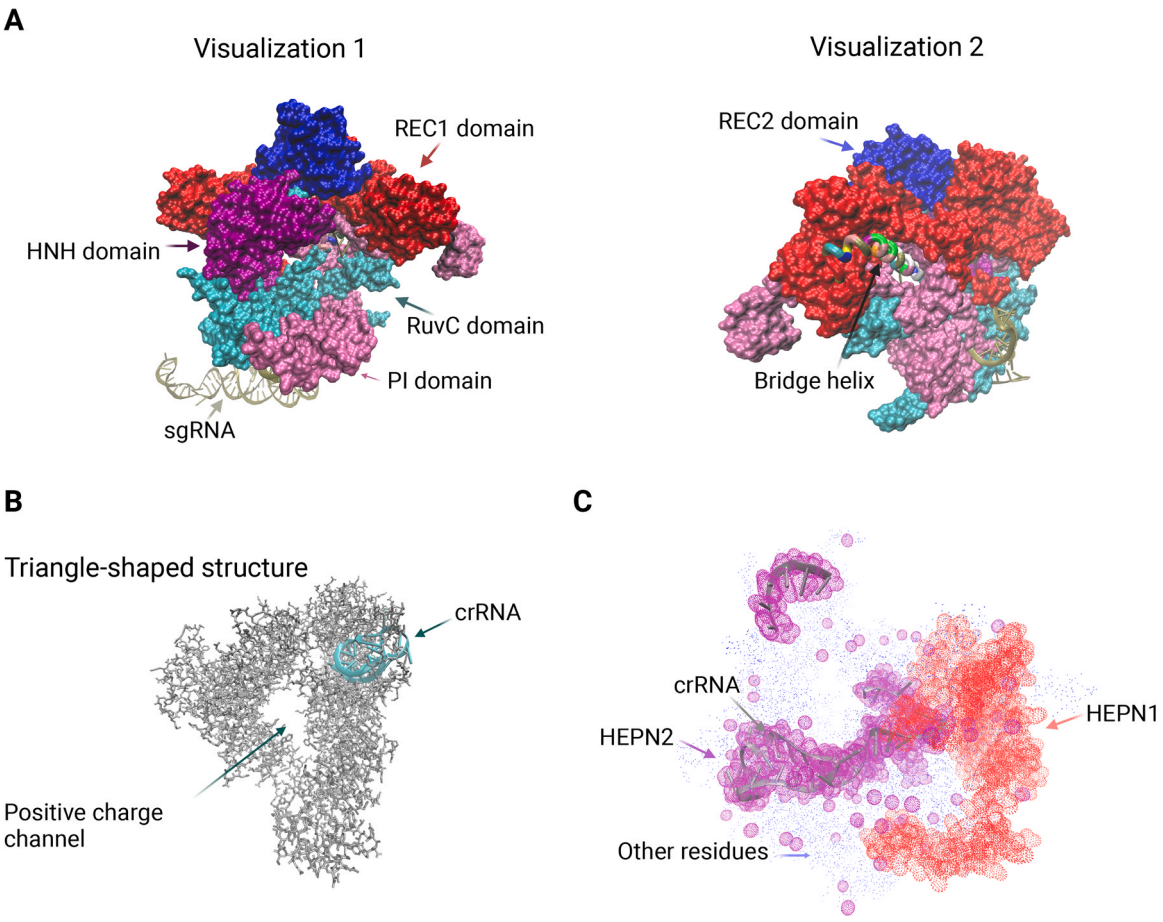

Fig. 1. The crystal structure of Cas protein complexes. (A) The sgRNA/SpCas9 complex. (B) The crRNA/LbCas12a complex. (C) The crRNA/LshCas13a complex.

subtypes, namely Cas12a and Cas12b. It is a single large Cas protein with a REC lobe and a NUC lobe, similar to Cas9.

Cas12a from *Lachnospiraceae bacterium* (LbCas12a/LbCpf1 PDB code: 5ID6) is well characterized by a triangle-shaped structure in which a large positive charge channel is located in the center (Fig. 1B) [28]. Though closely comparable in size (SpCas9: 1369 amino acids (aa), LbCpf1: 1274 aa), LbCpf1 works by a very different mechanism to SpCas9, relying on a significantly smaller crRNA that contains only 42–44 nucleotides and requires no complementary base pairing with a tracrRNA, which is required by the crRNA of SpCas9 [28,29]. The crRNA binds to the oligonucleotide binding domain of LbCpf1 and induces an evident structural rearrangement of LpCpf1, which results in the formation of the LbCpf1 substrate binding conformation, setting the stage for binding to potential target dsDNA [28]. Moreover, Cas12a inhibitors such as type V-A anti-CRISPRs (AcrVAs), which are mobile genetic elements that insert the binding pocket for the PAM of LpCpf1 by mimicking the PAM sequence, can cause crRNA degradation in a Cas12a-dependent manner and in turn induce complete inactivation of cleavage of LpCpf1 for foreign complementary dsDNA [30].

The activity described above, which depends strongly on crRNA and cleaves its complementary paired dsDNA, is referred to as *cis*-cleavage activity. In the pathogen detection application of Cas12a, a function known as *trans*-cleavage, which occurs after *cis* cleavage, was more commonly employed. Following recognition of the PAM, the crRNA hybridizes with the target dsDNA and triggers conformational activation to achieve exposure of the RuvC catalytic site, causing *cis* cleavage of the dsDNA [31,32]. After *cis* cleavage, the PAM-proximal fragment of target dsDNA (referred to as an activator DNA) is still remained by Cas12a, which preserves the exposure of the RuvC domain. RuvC structural domain, maintaining its catalytic activity, is free to cleave any single strand DNA (ssDNA), ssRNA, and dsDNA suitable for its catalytic pocket in *trans*, independent of sequence [33,34]. Since *trans*-cleavage activity of Cas12a avoids PAM and sequence restrictions, freely designable oligonucleotides became effective reporter molecules and were widely used in pathogen detection.

The other important subtype of Cas12, Cas12b, has also attracted much research interest, particularly the one identified from *Alicyclobacillus acidoterrestris* (AacCas12b/AacC2c1). Compared with SpCas9 and LbCpf1, AacC2c1 exhibits a smaller size (1129 aa), but both possess a bilobed structure composed of REC and NUC lobes [35,36]. In terms of working mechanisms, AacC2c1 targets dsDNA relying on a dual-RNA guide, with crRNA located in its central channel and tracrRNA located in the external surface groove. [36,37]. What is impressive about AacC2c1 is the high specificity of dsDNA cleavage and low tolerance of single nucleotide mismatches between guide RNA and target dsDNA which makes it highly accurate for the direct detection of pathogenic dsDNA [38].

2.3. Cas13

Cas13 is the signature protein of type VI Cas systems in the form of single large protein like Cas9 and Cas12. Among the vast kingdom of bacteria, Cas13a (C2c2) found from *Leptotrichia shahii* (LshCas13a), *Leptotrichia buccalis* (LbuCas13a), *Lachnospiraceae bacterium* (LbaCas13a), *Leptotrichia wadei* (LwaCas13a), *Capnocytophaga canimorsus* (CcaCas13b), and *Prevotella sp.* (PsmCas13b) are currently better understood [39].

Structure studies of LshCas13a (PDB code: 5WTK), and LbuCas13a (PDB code: 5XWP) have revealed that Cas13a distinguishes itself structurally from Cas9 and Cas12 and targets entirely differently to both,

being RNA rather than DNA [40,41]. More specifically, Cas13a contains 2 higher eukaryotes and prokaryotes nucleotide-binding domains (HEPNs) instead of the HNH and RuvC domains used by Cas9 and Cas12, to enable cleavage specificity for RNA targets (Fig. 1C) [42,43]. Moreover, similar to Cas9, mutations in key residues in the Cas13a molecule can form a nuclease activity-deficient Cas13a (dCas13a), which is able to bind RNA targets but unable to cleave them [44]. Interestingly, HEPN is functionally unique in that once it recognizes and cleaves the RNA target specified by the crRNA sequence, it transitions into an enzymatic *trans*-state where it binds and cleaves other RNAs non-specifically at non-specific sites where rich in uridine, triggering programmed cell death or dormant state in bacteria [43,45]. This is the strategy adopted by bacteria in their natural struggle against phages, which aims to limit the spread of infection throughout the population at the expense of self. Unsurprisingly, the flexible substrate selection of *trans*-activity from the Cas13a HEPN domains constitutes an invaluable basis for the detection of pathogenic nucleic acids based on the CRISPR/Cas system.

The structure insights of Cas13b mainly concentrate on *Bergeyella zoohelcum* Cas13b (BzCas13b) and *Prevotella buccae* Cas13b (PbuCas13b) [46,47]. Most structural regions of both BzCas13b and PbuCas13b are distinctly different, except for 2 HEPNs conserved in classical structure [46,47]. The structure of BzCas13b is assumed to be triangular domains distributed around a central L-shaped crRNA, while domains of PbuCas13 are distributed across its linear domain architecture in a unique arrangement [46,47]. More recently a Cas13 subtype that have been utilized for pathogen detection is Cas13d, such as the one derived from *Ruminococcus flavefaciens* (CasRx) [48]. Although CasRx is already being applied to detect pathogens, its structure has not been precisely determined. Structure analysis of Cas13d from *Eubacterium siraeum* (EsCas13d) may be able to contribute some insights that Cas13d also contains 2 conserved HEPNs that migrate towards each other within the HEPN1 and HEPN2 domains to produce an externally oriented active site, which is favorable for direct hydrolysis to cleave complementary target RNAs in *cis* and non-complementary RNAs in *trans* [49].

2.4. Other Cas proteins

2.4.1. Cas14

Cas14 (Cas12f) proteins are also type V Cas proteins that have a markedly smaller size (only 400–700 aa), only half that of the Cas9 protein, which makes it potentially more amenable to transfection and expression [50]. Functional studies have shown that the Cas14a protein performs cleavage of ssDNA through the complementary pairing of partner sgRNA with the substrate ssDNA without PAM restriction that provides Cas14a with a greater scope for sequence selection [50]. Also, Cas14a can bind to the other Cas14a to form an asymmetric Cas14 dimer with 2 sgRNAs for recognition and cleavage of dsDNA substrates with T-rich PAM sequences [51–53]. In addition, Cas14a has a poor tolerance for mismatches near intermediate sequences, which has led to the development of the capability of Cas14a to detect DNA single-nucleotide polymorphisms (SNPs). Similar to Cas12 and Cas13, Cas14 triggers nonspecific trans-cutting after cis recognition with the substrate of ssDNA [50].

2.4.2. Cas3

Cas3 is the signature nuclease of the CRISPR/Cas type I system, which is the most common type of CRISPR in bacteria, accounting for 95% of cases [54–56]. The competence of Cas3 for gene editing in eukaryotic cells was discovered only after type II, type V, and type VI Cas nucleases. Surprisingly, Cas3 derived from *Thermomonaspora fusca* was proven to enable the knockout of large DNA segments in human embryonic stem cells, ranging from a few hundred base-pairs to 100 kilobases, as it not only causes DSB, but eliminates DNA fragments sequentially [57,58]. The type I CRISPR/Cas system is also unique in respect of gene regulation. As the type I CRISPR Cas system is a multi-protein system containing the multi-component ribonucleoprotein complex called Cascade (CRISPR-associated complex for antiviral defense) and Cas3 in which the former is dedicated to identifying and binding target dsDNA, while the latter is only responsible for degrading nucleic acids, deleting Cas3 allows Cascade to tightly bind target dsDNA sequences without recruiting Cas3 and degradation [54,59-61]. Solid sequence occupancy by Cascade prevents RNA polymerase recruitment or extension thus realizing gene expression repression. It can be seen that the multi-protein type I CRISPR/Cas system with the Cas3 targeting large segments of DNA is ill-suited and less desirable for precise gene editing, but is of great potential for some extended applications.

2.4.3. Cas10

Cas10, the signature protein of the CRISPR/Cas type III system, not only demonstrates trans-acting DNase activity, but also serves as an adenylyl cyclase and DNA polymerase [62–65]. Once the invading DNA is undamaged by type I and II CRISPR/Cas systems and heavily transcribed, the DNase activity exerted by the Cas10 HD domain and RNase activity induced by the Cas10 Palm domains will be activated to indiscriminately cleaves exogenous and endogenous DNA as well as RNA [62,66–70]. Due to its multiple cleavage activity, Cas10 has also been successfully used to detect pathogens [71].

2.4.4. Csf1

Csf1 is the signature protein of the CRISPR/Cas type IV system, which is highly diverse in structure. A structural study of the type IV-B CRISPR-Cas RNP complex reveals that the complex does not assemble with crRNA, but rather on regular lengths of heterogeneous RNA arranged as pseudo-A form, which makes it distinct from other types of CRISPR/Cas systems [72]. As the structure and mechanism of the type IV CRISPR/Cas system still need to be further explored, there are currently no reports of it being used for pathogen detection.

3. HIV detection by CRISPR/Cas systems

HIV testing mainly refers to the qualification and quantification of HIV antibodies, proteins, and nucleic acids, in which both proviral DNA and viral RNA can serve as the target. In 2016, the Prevention Access Campaign came up with the slogan U═U (undetectable equals untransmittable), which means that a person living with HIV who is on treatment and has an undetectable viral load cannot sexually transmit HIV [73]. This conclusion has been accepted by the World Health Organization, as well as many other leading medical organizations. The scientific validity of this conclusion has also been demonstrated in a number of relevant clinical trials, such as the PARTNER study and the HPTN 052 trial, both of which found that virologically-suppressed carriers (plasma HIV-1 RNA $\leq$ 200 copies/mL) are incapable of sexually transmitting the virus, making it clear that control of the HIV epidemic is closely linked to effective HIV detection [74,75].

Although the conclusion that U═U has gained much acceptance, the expensive and high technological threshold PCR nucleic acid testing still prevents it from being well generalized. More POC-amenable tests could better facilitate HIV nucleic acid testing in remote and underdeveloped areas, help a wider range of people living with HIV to know their status in a timely manner, and contribute to the elimination of stigma and discrimination against HIV-positive people. Utilizing CRISPR-based approaches for HIV testing could provide rapid, affordable, accessible, and precise alternatives. These methods aim at detecting DNA or/and RNA of HIV and underscore inclusiveness and universality of the CRISPR/Cas system by combining it with different tools, elements or platforms (Table 2).

3.1. HIV RNA detection based on CRISPR/Cas system

HIV genome consists of two identical viral single-strand, positive-sense RNAs. Thus, detecting HIV RNA directly reflect the viral quality and quantity. However, to test for HIV RNA, types of CRISPR/Cas system

Table 2
CRISPR-based method characteristics.

Target	Method (Cas protein)	Amplification assay	Sample type, LOD, and assay time	Additional elements (portable or not)	Brief assay steps
HIV RNA	Cas9 nAR-v2 (SpCas9)	Cas9n-assisted isothermal amplification reaction	1.2 copies /reaction for synthetic HIV RNA without direct reference to total assay time	No specific additional elements required	1. Mix and incubate the kit of Cas9 nAR-v2 2. Add the mixture to a lateral flow strip
	rRT-RAA-Cas12a (LbCas12a)	rRT-RAA	2 copies /reaction within 30 min for synthetic HIV RNA	No specific additional elements required	1. Add HIV RNA and magnesium acetate to trigger rRT-RAA reaction 2. Add crRNA/Cas12a complex and ssDNA-FQ reporter to the mixture 3. Detect by a fluorescence reader
	CRISPR gel (LbCas12a)	None	30 copies/reaction within 30 min for synthetic HIV RNA	Low-melting point agarose gel (portable as fit in a tube)	1. Add RT-RPA reaction solution to the CRISPR gel 2. Incubate the mixture at 42∘C for isothermal detection 3. Detect by naked eyes
	Smartphone-enabled digital CRISPR assay (LbCas12a)	RT-RPA	75 copies/reaction within 15 min for synthetic HIV RNA	Smartphone-based platform (portable)	1. Load HIV RNA and RPA buffer mixture into the QuantStudio chip and insert the platform 2. Incubate at 42∘C for isothermal detection 3. Acquit fluorescence by the platform and analyze by Deep learning
	CARMEN-Cas13 (LwCas13a)	PCR or RPA	10^{-18} M without direct reference to total assay for synthetic HIV RNA	Color code, microwell array (not portable)	1. Amplify, color code, and emulsify samples 2. Add samples into microwells and image color code by fluorescence microscopy 3. Electric merge droplets and monitor microwells by fluorescence microscopy
	STAMP-dCRISPR (LwCas13a)	None	2000 copies/mL within 1 h for HIV viral particles spiked in plasma	STAMP device (portable)	1. Extract HIV RNA from plasma 2. Mix HIV RNA and the Cas 13a reaction mixture and take in the STAMP device 3. Image by fluorescence microscopy
HIV DNA	CRISPR/Cas12a coupled with enzyme-DNA molecular switch PEC assay (LbCas12a)	Enzyme-DNA molecular switched amplification	0.3 fM without direct reference to total assay time for synthetic HIV DNA added to serum	Enzyme-DNA molecular switch (portable as fit in a tube)	1. Make molecular switches 2. Mix crRNA/Cas12a complex, sample, and switches 3. Magnetically separate and detect by the ZrCNSs-modified gold electrode
	AuNPs-tagging-based CRISPR-Cas12a bioassay platform (LbCas12a)	None	1.05 amol/reaction within 40 min for synthetic HIV DNA	AuNP, ICP-MS (not portable)	1. Mix and incubate AuNPs-ssDNAs-MBs and crRNA/Cas12a complex 2. Magnetically separate and detect by ICP-MS
	SCAN (LbCas12a)	None	10 nM within 1 h for synthetic HIV DNA	Solid-state nanopore, 6363 DAQ card (portable)	1. Add HIV DNA and CRISPR/Cas12a reaction mixture 2. Drive the mixture into nanopores by a constant voltage and acquit current by the 6363 DAQ card
	CRISPR-SERS-LFA (LbCas12a)	None	0.3 fM within 1 h synthetic HIV DNA	Lateral flow strip and Raman spectrometer (not portable)	1. Mix Cas12a/crRNA complexes, probes, and HIV DNA 2. Add mixture to a SERS-strip 3. Detect by a Raman spectrometer
	CRISPR-Cas12a-PCHA (LbCas12a)	None	4.2 fM with 120 min for synthetic HIV DNA	Nucleic acid hairpins (portable as fit in a tube)	1. Mix HIV DNA and CRISPR/Cas12a complex and H0-MB 2. Magnetically separate and add H2 and H3 3. Image by Fluorescence Spectrophotometer
	PEC-CRISPR/Cas12a assay (LbCas12a)	None	0.4 fM without direct reference to total assay time for synthetic HIV DNA	Nucleic acid hairpins, SA-ALP, and (AuNPs)/MoO2/Mo2C-modified electrode (not portable)	1. Sequentially mix HIV DNA, H1-MB, H2-biotin, and SA-ALP for incubation 2. Magnetic separate and sequentially add crRNA/Cas12a complex, p-APP, and $Ru(bpy)_3^{2+}$ 3. Immerse AuNPs/MoO_2/Mo_2C-modified electrode in the mixture for detection
	CRISPR-PGM platform (LbCas12a)	None	11 fM without direct reference to total assay time for synthetic HIV DNA	Invertase catalytic mix. PGM (portable)	1. Mix crRNA/Cas12a complex, DNA-invertase probe, and HIV DNA and cool the mixture 2. Detect by a commercial PGM

(*continued on next page*)

Table 2 (*continued*)

Target	Method (Cas protein)	Amplification assay	Sample type, LOD, and assay time	Additional elements (portable or not)	Brief assay steps
	Dual-QD-dCas9 (SpCas9)	None	1 copy/cell without direct reference to total assay time	QD (not available)	1. Make CRISPR-dCas9-QD625 and CRISPR-dCas9-QD525 2. Transfect into U1 cell lines 3. Image by fluorescence microscopy
Both HIV DNA and RNA	AIOD-CRISPR (LbCas12a)	RPA or RT-RPA	1.2 copies/reaction for synthetic HIV DNA and 11 copies/reaction for synthetic HIV RNA within 40 min	No specific additional elements required	1. Sequentially mix (RT)-RPA buffer, ssDNA-FQ reporters and crRNA/Cas12a complex for incubation 2. Visually detect by using a LED blue light illuminator
	CRISPR-MCR (LbCas12a)	RPA or RT-RPA	43 copies/reaction for synthetic HIV DNA and 200 copies/reaction for synthetic HIV RNA within 30 min	Microfluidic platform, nanoporous PES membrane, invertase, and PGM (portable)	1. Incubate the biosensor at 40 ∘C for isothermal detection 2. Press the water blister and incubate at 40 °C for the invertase catalysis 3. Detect by the PGM

primarily targeting DNA require incorporating a reverse transcription step.

3.1.1. *Cas9 nAR-v2*

A method named Cas9 **n**ickase-based **a**mplification **r**eaction **v**ersion **2** (Cas9 nAR-v2) was utilized to detect HIV RNA [76].As mentioned earlier, Cas9n with inactivation of the RuvC domain or the HNH domain can cut only one strand of the target DNA, i.e., resulting in an SSN, to be used as a starting point for strand displacement amplification (SDA), an isothermal amplification reaction based on exonuclease-deficient Klenow (exo$^-$ Klenow) DNA polymerase to extend the nick site and replace the downstream strand [24,77]. However, Cas9n's excessive affinity for the nicked dsDNA target greatly hinders the DNA polymerase from binding to the nicked DNA site, resulting in a difficult and inefficient amplification reaction [78]. To address this problem, the researchers introduced amino acid mutations at multiple sites in different domains of Cas9n based on structure-guided protein engineering. These selected amino acid residues, 16 in number, play an important role in the interaction of the sgRNA/Cas9n complex with the nicked dsDNA and in stabilizing the phosphate backbone of the nicked dsDNA [79–81]. Exchanging them to alanine or glutamate caused varying degrees of affinity changes between Cas9n and the nicked dsDNA, and finally a Cas9n variant that dissociates fastest from dsDNA (termed fastCas9n) being used as a tool enzyme for Cas9 nAR-v2 [76,79,81].

The kit of Cas9 nAR-v2 for HIV RNA detection contains reverse transcriptase, exo$^-$ Klenow DNA polymerase, 3 sgRNA: Cas9n complexes, 2 primers, and SYBR Green, which are homogenously mixed with the synthesized HIV RNA and incubated at a constant temperature [76]. The working principle can be briefly described that after reverse transcription for forming the HIV RNA: cDNA hybrid complex and subsequently RNase H digestion of HIV RNA, the first ssDNA is synthesized using the HIV cDNA as template for forming the HIV cDNA: the first ssDNA complex. Then sgRNA1: Cas9n and sgRNA2: Cas9n each create an SSN in the first ssDNA, causing the first ssDNA to be released by exo$^-$ Klenow DNA polymerase through the strand displacement activity (Fig. 2) [76,77]. The first ssDNA is then used as a template for the second ssDNA, which is next released in cooperation with exo$^-$ Klenow DNA polymerase and sgRNA3: Cas9n. The second ssDNA is afterward used as a template, generating the first ssDNA in turn. The first ssDNA and the second ssDNA are used as templates to generate each other cyclically, resulting in the rapid production of numerous DNA fragments and thus binding the SYBR Greens for fluorescence detection. The limit of detection (LOD) of HIV RNA by Cas9 nAR-v2 was 10^{-8} ng/20 μl [76]. Besides, reporting signals by fluorescence, the additional modification of carboxyfluorescein (FAM) and biotin moieties in primer 1 and primer 2, respectively, allow the implementation of Cas9 nAR-v2 in a

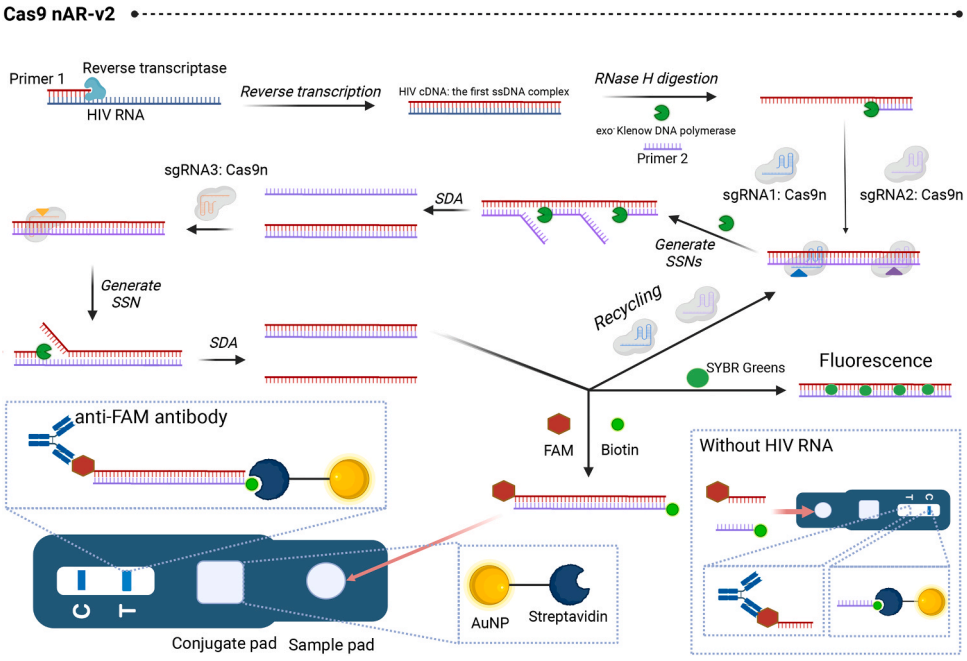

Fig. 2. Cas9 nAR-v2 kit combining with a lateral flow strip.

commercially available lateral flow strip, whose conjugate plate is coupled with AuNP-streptavidin, as well as the test line and the control line is immobilized with anti-FAM antibodies and biotin-secondary antibodies, respectively. When the reaction mixture is added dropwise to the sample plate, the amplified DNA fragments are coupled by the AuNP-streptavidin through biotins and then captured by the anti-FAM antibodies on the test line, resulting in aggregation of a large number of AuNP that produces a red line visible to the naked eye. Without HIV RNA, AuNPs will be captured by biotin-secondary antibodies and accumulate in the control line. In a word, combining the Cas9 nAR-v2 and the lateral flow strip makes this method the potential to be applied in scenarios of low-cost POC test [76].

3.1.2. rRT-RAA-Cas12a

Not only Cas9-related variants, Cas12a is also used to detect HIV RNA. The Cas12a-based detection scheme likewise uses the idea of coupling with isothermal amplification techniques, but the coupling technique is **r**eal time **r**everse-**t**ranscription (RT) **r**ecombinase-**a**ided **a**mplification (rRT-RAA) instead of SDA [7]. It is important to clarify that the rRT-RAA is already effective in detecting HIV on its own, only when the addition of Cas12a further enhances the efficacy of the test is it a reasonable combination strategy, otherwise it will only result in redundancy of operation and a waste of resources. Therefore, the researchers performed rRT-RAA and rRT-RAA-Cas12a on purified HIV RNA detection separately.

The essential proteins included in the rRT-RAA kit are UvsX recombinase, single-strand binding protein (SSB), DNA polymerase, and reverse transcriptase [82,83]. The principle can be briefly described as follows, under the isothermal condition (39–42 °C), after reverse transcription of the HIV RNA to form the HIV RNA/cDNA heteroduplex, the UvsX recombinase binds to the primer to form a polymer, which unwinds the HIV RNA/cDNA heteroduplex at the sites homologous to the primer (Fig. 3) [82]. Next, SSB binds and stabilizes HIV RNA, and meanwhile, polymerase catalyzes strand extension to form dsDNA. The generated dsDNA serves as a new substrate to be invaded by the UvsX recombinase/primer polymer to initiate a new round of amplification and sequential cycling [83]. The LOD for HIV RNA using the rRT-RAA alone was 20 copies per reaction (~20μl) [7]. It could also differentiate HIV genotypes such as CRF01_AE, CRF07_BC, and others.

In order for Cas12a to associate smoothly with rRT-RAA, the generated dsDNA by rRT-RAA can bind specifically to the designed crRNA. Guided by crRNA against product dsDNA, Cas12a can be rapidly and efficiently activated [7]. If the product is not HIV DNA, but of another pathogen such as hepatitis B or C virus, Cas12a will not exert its *cis*-cleavage, showing excellent specificity [7]. Following *cis*-cleavage, *trans*-cleavage is performed on the additionally added ssDNA-FQ reporter by the Cas12a RuvC domain that remains exposed and active [33,34]. The exposed RuvC domain cut the ssDNA, resulting fluorescence produced by FAM isolated from black hole quencher (BHQ) can be observed by the naked eye using a blue light imager, allowing it to be a suitable on-site testing method. The combined rRT-RAA-Cas12a method involves two signal amplification processes, the first being the bulk generation of dsDNA substrate by rRT-RAA to activate enough Cas12a, and the second being the self-amplification of the signal by Cas12a, for one Cas12a can cleave multiple ssDNA-FQ reporters, resulting in the release of multiple FAMs. All components used in this method are added sequentially to the single, one-pot reaction system, avoiding the possibility of contamination during rRT-RAA product transfer. In this study, rRT-RAA-Cas12a was able to detect 2 copies of purified HIV RNA per reaction within 30 min, which is an order of magnitude higher than the LOD of the rRT-RAA alone.

3.1.3. CRISPR gel

CRISPR gel is also a CRISPR/Cas12a-based method that sequentially completes all reactions into a one-pot system, but differs from the previous method (rRT-RAA-Cas12a) in that it uses an isothermal

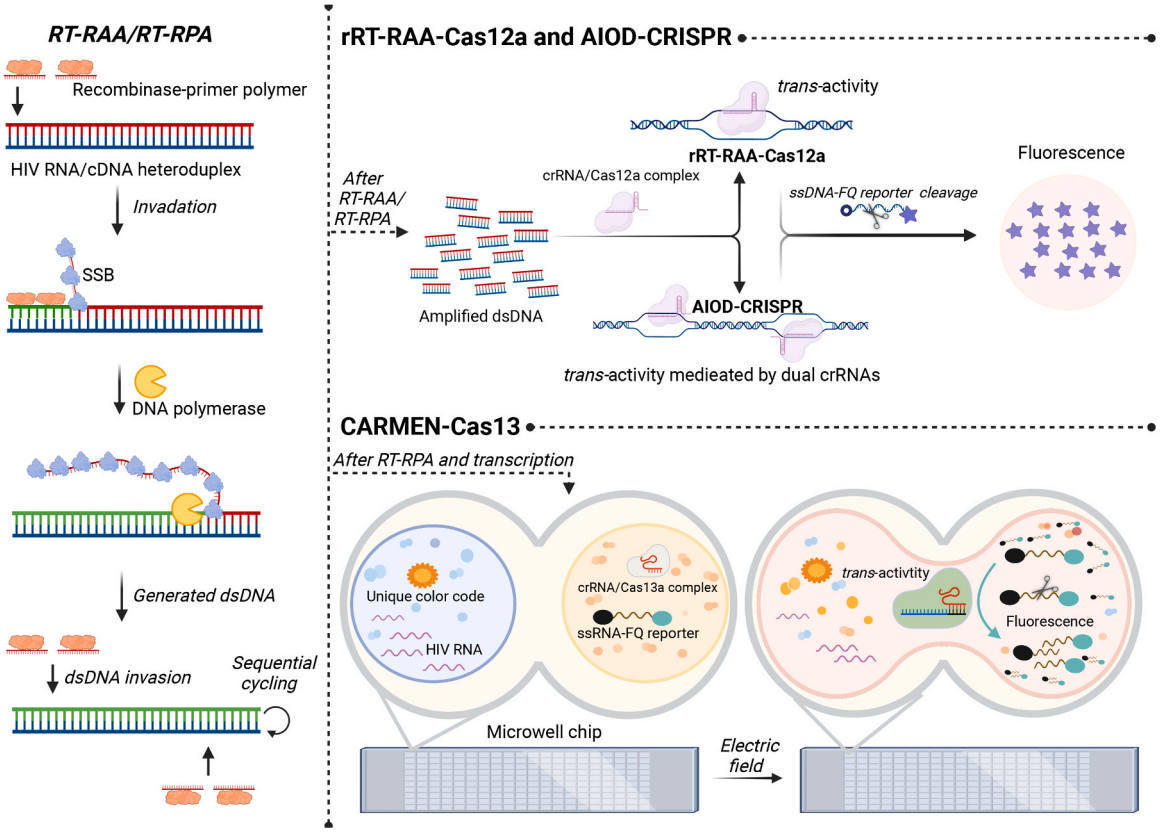

Fig. 3. rRT-RAA-Cas12a and AIOD-CRISPR and CARMEN-Cas13. These methods are combined with RT-RAA or RT-RPA.

amplification technique called reverse-transcription recombinase polymerase amplification (RT-RPA), which shares the same principle as RAA but uses a different source of the recombinant enzyme [84].

This method allows the reactions to be integrated in a single tube and is characterized by the use of agarose gel to spatially separate the CRISPR reaction solution from the RT-RPA reaction solution, which can effectively improve their compatibility [84]. For the preparation, the researchers initially mix the CRISPR reaction solution with low-melting point agarose gel in one tube and placed the mixture on ice to generate the CRISPR gel, followed by the addition of RT-RPA reaction solution, which would not merge with the CRISPR reaction solution due to the presence of agarose gel (Fig. 4).

When the samples are tested, the optimized temperature (42 °C) required for the RT-RPA reaction melts the low-melting point agarose gel, resulting in the mixing of the RT-RPA reaction with the CRISPR reaction solution. RT-RPA amplicons diffuse into the gel and initiate the CRISPR reaction, causing ssDNA-FQ reporters to be cleaved by Cas12a's *trans*-activity to produce fluorescence visible to the naked eye. With the CRISPR gel biosensing platform, the researchers successfully detected down to 30 copies of HIV RNA per test within 30 min [84].

3.1.4. Smartphone-enabled digital CRISPR assay

An interesting study integrated RT-RPA and CRISPR/Cas12a reactions in a commercial stamp-sized digital chip (QuantStudio digital chip) and enabled the presentation of HIV RNA detection results on a smartphone [85]. To this end, the researchers designed a portable smartphone-based platform that allows for temperature and analysis time control, as well as imaging of fluorescence via the smartphone camera (Fig. 5) [85].

The workflow of the device Is not complicated: after a QuantStudio digital chip loaded with a solution consisting of the sample, RT-RPA, and the CRISPR/Cas12a reaction system is inserted into the platform, RT-RPA occurs at a set constant temperature (42 °C), generating a large number of amplicons to activate CRISPR/Cas12a trans- activation, causing ssDNA-FQ reporters to cut and release fluorescence, which is monitored and recorded in real time by the smartphone's camera [85,86]. And then, the fluorescence images were analyzed by a trained and validated deep learning-based algorithm to quantify HIV RNA [87]. After optimization, the device can detect 75 copies of HIV RNA within 15 min.

Although the detection sensitivity of the smartphone-based platform device is not ideal, the user-friendliness of the operation and data interpretation, and the significantly lower price (thousands of dollars) compared to commercial or research devices, make it a promising tool for POC testing [85].

3.1.5. CARMEN-Cas13

Based on Cas13a's sensitive detection of RNA rather than DNA, a technique known as **c**ombinatorial **a**rrayed **r**eactions for **m**ultiplexed **e**valuation of **n**ucleic acids (CARMEN)-Cas13 has been developed to detect HIV RNA [88,89]. The technique is a valid extension of a protocol called specific high sensitivity enzymatic reporter unlocking (SHERLOCK), which used isothermal amplification and CRISPR/Cas13 system to detect the nucleic acid of Zika and Dengue virus to offer a rapid, inexpensive, and sensitive nucleic acid detection alternative for POC test [90]. The workflow of SHERLOCK is very similar to that of rRT-RAA-Cas12a, i.e., after the nucleic acid has been amplified by RPA or RT-RPA and transcribed by T7 RNA polymerase, the product specifically activates the *cis*- and subsequent *trans*-activities of Cas13a, leading to cleavage of the reporter molecules and release of the fluorescent groups that enable the qualification of the targeted nucleic acid [90].

Over SHERLOCK, the main improvement of CARMEN-Cas13 is the significantly higher throughput, allowing simultaneous detection of up to 169 viruses, including HIV. On the CARMEN-Cas13 platform, each pre-amplified sample is mixed with a unique color code consisting of four fluorescent dyes in a ratio of 1050 combinations (Fig. 3) [88]. Next, each sample with a different fluorescent color is individually emulsified with oil and then added to a separate microwell on a chip. As a

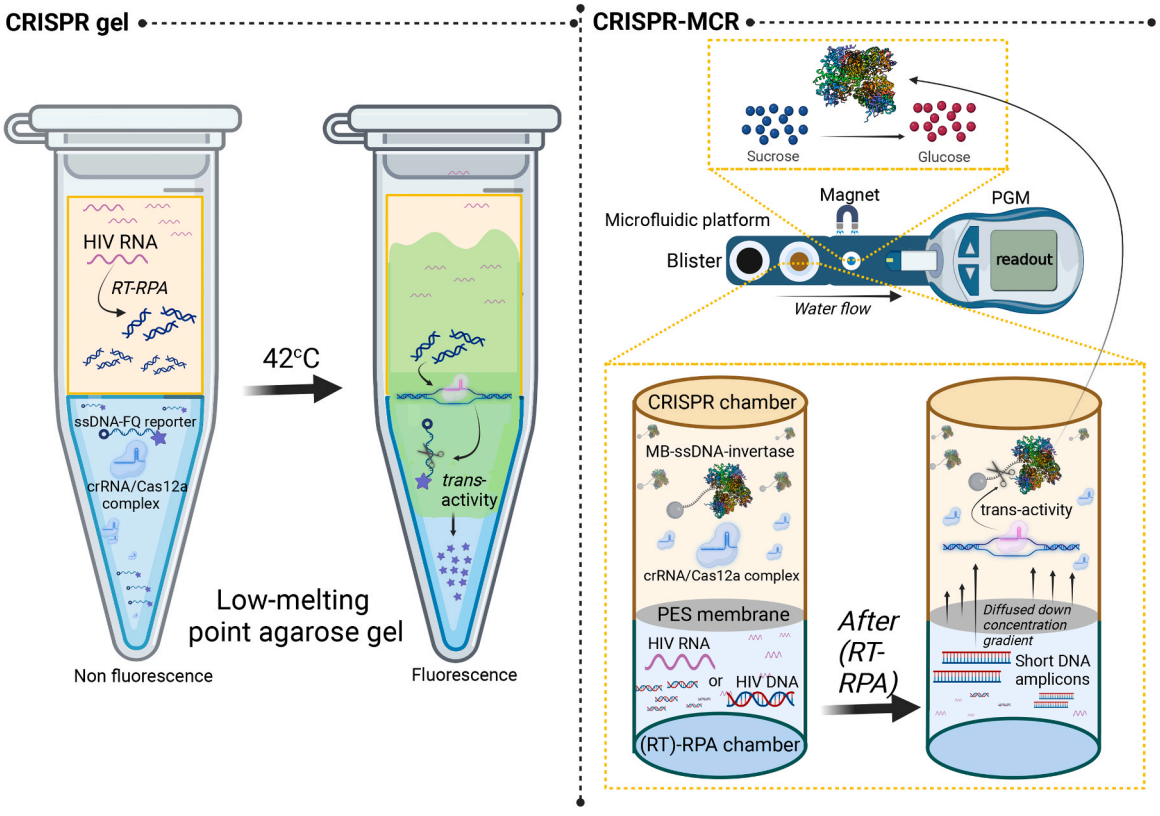

Fig. 4. CRISPR gel and CRISPR-MCR. These methods employ the strategy of spatial separation.

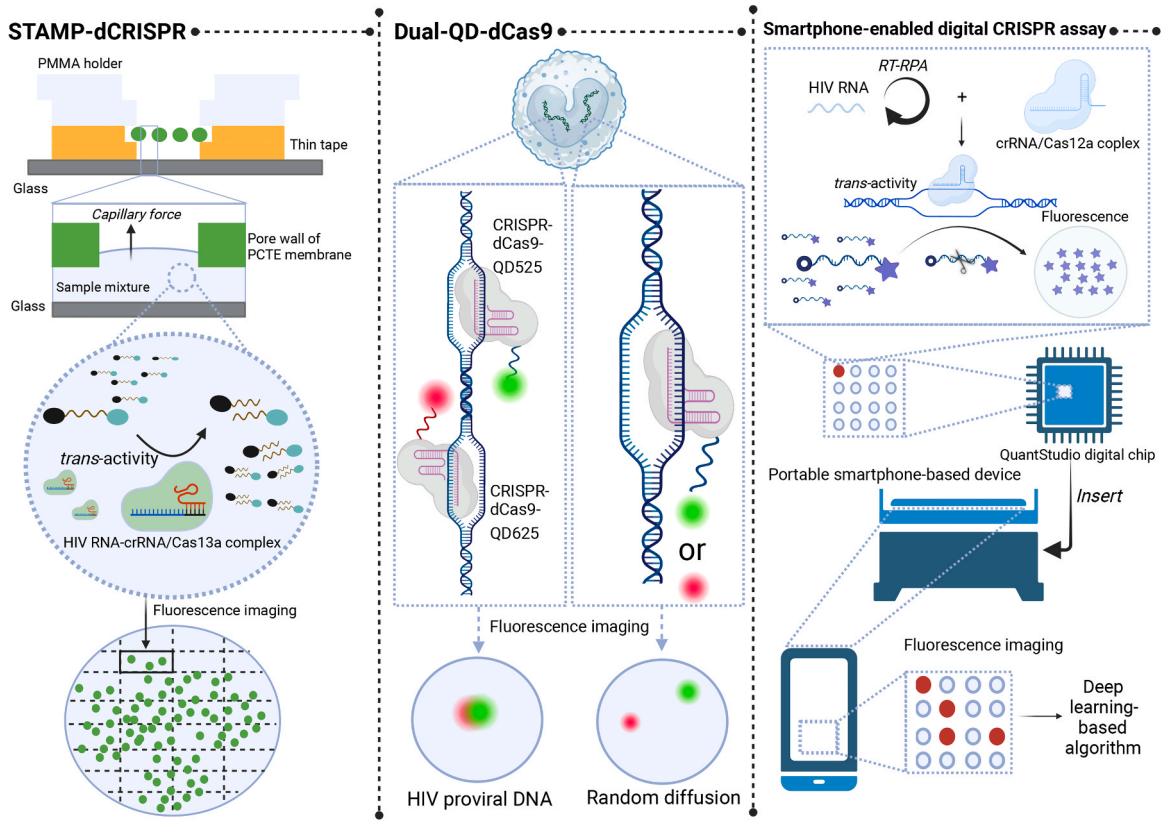

Fig. 5. STAMP-dCRISPR and Dual-QD-dCas9 and Smartphone-enabled digital CRISPR assay. These methods provide on-screen visualization of HIV.

microwell can only hold two droplets, one of which contains pre-amplified nucleic acids and color-identifier dye, and the other one contains the crRNA/Cas13a complex and ssRNA-FQ reporters, a tiny reaction system is formed by mixing the two droplets through exposure to an electric field which can be identified by its unique fluorescent color. Upon recognition of the sample by CRISPR/Cas13a, *trans*--cleavage is activated, resulting in the cleavage of ssRNA-FQ reporters and the release of fluorescence for detection. It is worth mentioning that the CARMEN-Cas13 platform can also identify 6 different drug-resistance mutations in HIV at once, because of the exquisite specificity of Cas13. Although the ability of CARMEN-Cas13 to simultaneously detect multiple pathogens is remarkable, its reliance on complex equipment and high technical analysis thresholds restricts its potential as a POC test. To address this limitation, the implementation of more accessible equipment and integrated process may help CARMEN-Cas13 to fulfill the POC criteria.

3.1.6. STAMP-dCRISPR

Similar to CARMEN-Cas13, the self-digitization through automated membrane-based partitioning (STAMP) to digitalize the CRISPR-Cas13 assay (STAMP-dCRISPR) uses Cas13a and fluorescence imaging to detect HIV RNA, but unlike the former, STAMP-dCRISPR enables precise quantification of HIV RNA and eliminates the need for pre-amplification of nucleic acids, thus avoiding the potential risk of contamination associated with nucleic acid amplification [91].

The Fundamental to this approach is the invention of a device (i.e., STAMP) that digitizes the assay without the need for complicated fluidic control. Specifically, STAMP consists of a commercially available porous (pore sizes ranging from 10 nm to 30 μm) polycarbonate track-etched (PCTE) membrane sandwiched between a poly (methyl methacrylate) (PMMA) holder and a thin tape (Fig. 5) [92]. When STAMP is deposited on top of the sample mixture (including HIV RNA, CRISPR/Cas13 complex and ssRNA-FQ reporters) on the glass, the surface tension between the sample droplet and the pore wall induces capillary action, forcing the sample into and retained in the pores of the PCTE membrane. Followed by placing STAMP on a customized base with pre-filled mineral oil, a completely sealed digital system is constructed.

In the pores of the PCTE membrane, HIV RNA specifically binds to crRNA and activates CRISPR/Cas13 *cis*- and *trans*-activity, leading to cleavage of ssRNA-FQ reporters and release of fluorophores, each pore of which are recorded and integrated by fluorescence microscopy. The researchers adopted k-means clustering to identify positive pores containing HIV RNA and negative pores lacking HIV RNA, and the Poisson statistics to quantify the number of HIV-1 RNA targets, allowing for a LOD of approximately 2000 copies/mL HIV RNA [93].

3.2. HIV DNA detection based on CRISPR/Cas system

In common with other retroviruses, HIV RNA undergoes reverse transcription into HIV DNA upon invading a host cell. This process gives rise to the establishment of viral reservoirs, which constitutes a significant obstacle to HIV eradication efforts. Measuring the amount of HIV DNA provides insight into the magnitude of the viral reservoirs and aids in the clinical assessment of disease progression and drug efficacy.

3.2.1. CRISPR/Cas12a coupled with enzyme-DNA molecular switch photoelectrochemical (PEC) assay

This assay designed a novel molecular switch, an enzyme-DNA hybrid compound consisting of a Taq DNA polymerase, an aptamer that specifically binds to and inhibits the activity of Taq DNA polymerase, a MB-labelled block DNA paired with the aptamer, and a methylene blue-labelled primer paired with the block DNA (Fig. 6) [94,95].

When aptamer and block DNA are stably bound, aptamer effectively inhibits Taq DNA polymerase activity. In the event that HIV DNA is present in the reaction system, it will competitively bind block DNA

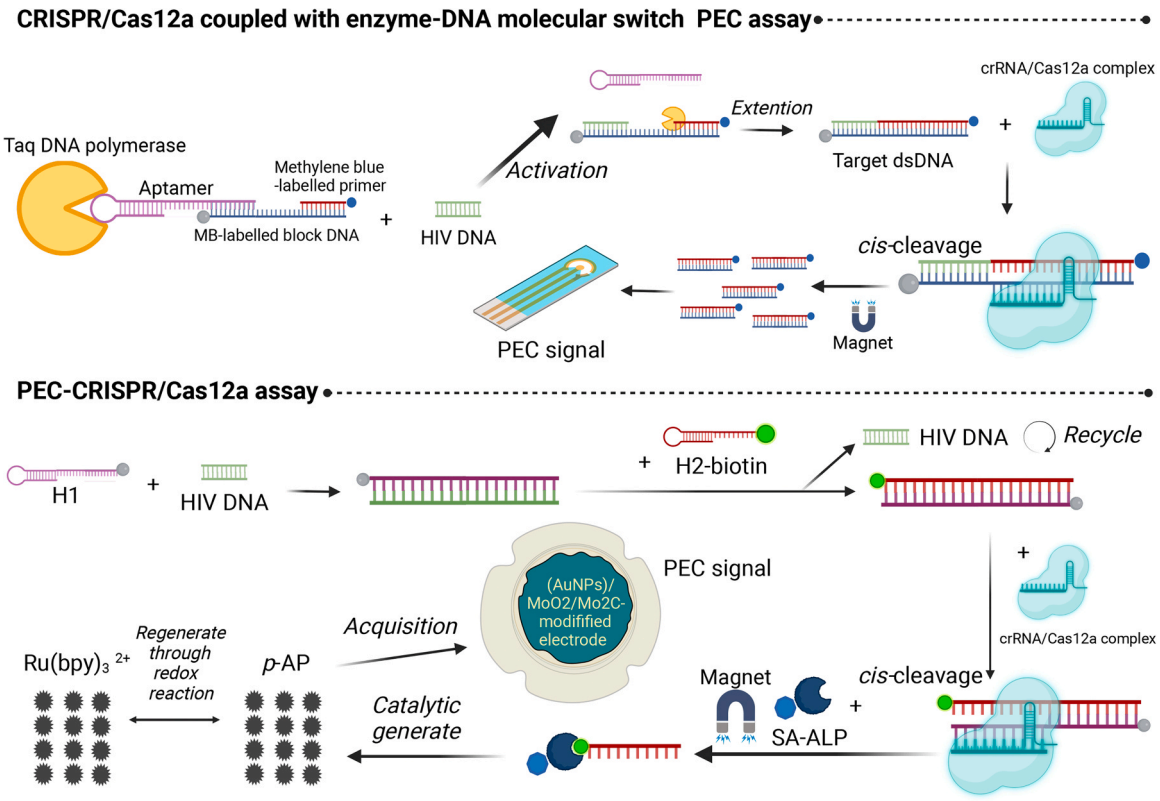

Fig. 6. CRISPR/Cas12a coupled with enzyme-DNA molecular switch PEC assay and PEC-CRISPR/Cas12a assay. These methods are based on *cis*-activity of Cas12a for HIV detection rather than *trans*-activity.

against aptamer, leading to the interruption of the stable binding of aptamer and block DNA, which triggers the activation of Taq DNA polymerase [96].

The activated Taq DNA polymerase extends the primer to generate sufficient amount of dsDNA, which is specifically recognized by the crRNA/Cas12a complex and cleaved by *cis*-activity. As a result, the methylene blue-labelled primer (or called dsDNA) is detached from the molecular switch, released from the magnetic adsorption, and free in solution, where it is obtained by the ZrCNSs-modified gold electrode to generate a PEC signal [97]. Under the best conditions, the assay achieved the LOD of HIV DNA as 0.3 fM.

3.2.2. *AuNPs-tagging-based CRISPR-Cas12a bioassay platform*

Although nucleic acid amplification based on DNA polymerase is effective in increasing signal, it also has disadvantages such as an increased risk of contamination. One study has invented a method called AuNPs-tagging based CRISPR/Cas12a bioassay platform by coupling the self-amplification effect of Cas12a with the enhancement effect of the gold nanoparticle (AuNP) to achieve nucleic acid amplification-free detection of HIV DNA [98]. AuNPs with scales of 1–100 nm have

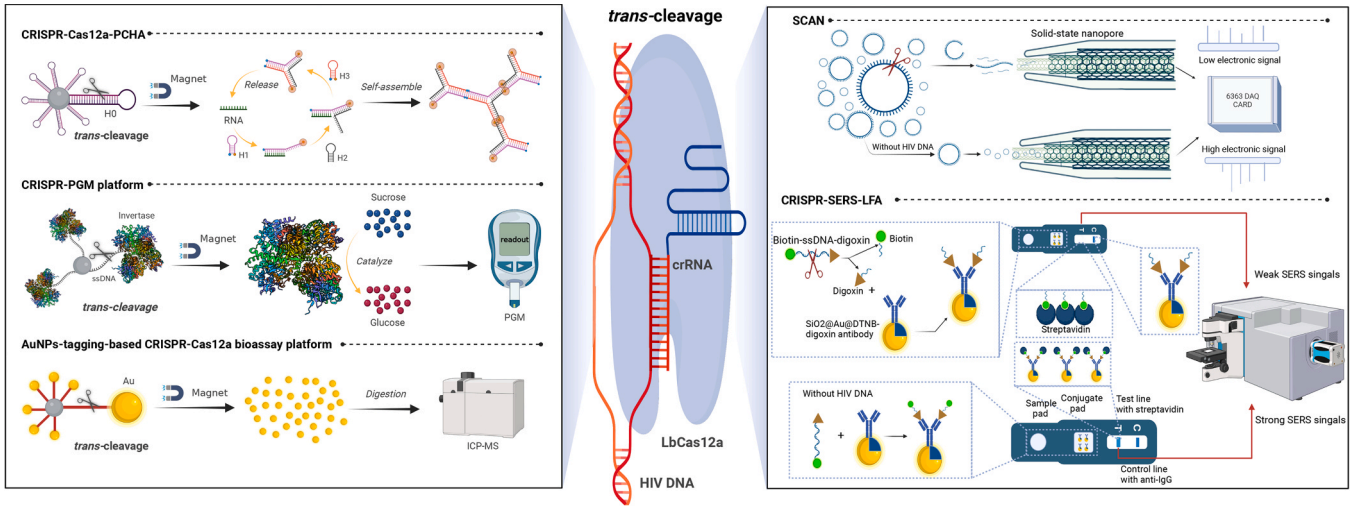

Fig. 7. CRISPR-Cas12a-PCHA, CRISPR-PGM platform, AuNPs-tagging-based CRISPR-Cas12a bioassay platform, SCAN, and CRISPR-SERS-LFA. These methods in which the crRNA/Cas12a complex is directly used to detect HIV DNA with involving magnetic separation (left) or not (right). The activation process of *trans*-cleavage of CRISPR/LbCas12a is shown in the center of the figure.

remarkable plasmonic properties and large surface-to-volume ratios, presenting features of high stability, biocompatibility, and ease of functionalization, making them widely used in biosensing platforms [99]. Since each AuNP contains a very high number of detectable atoms such as ^{197}Au, which allows it to be detected by inductively coupled plasma-mass spectrometry (ICP-MS) and produces a strong signal [98].

On the AuNPs-tagging-based CRISPR-Cas12a bioassay platform, the researchers used the signal generated by the AuNPs at ICP-MS to detect HIV. To correlate AuNP signaling with the presence of HIV, the researchers designed the ssDNA with a magnetic bead (MB) connected at the 5′-end and an AuNP tagged at the 3′-end, generating a AuNPs-ssDNAs-MB complex to be the reporter (Fig. 7). Upon HIV DNA detection by CRISPR/Cas12a complex, the *cis*-cleavage and *trans*-cleavage of Cas12a are activated in sequence, resulting in ssDNA probes being cleaved by Cas12a. Substantial amounts of AuNP are shed from the surface of the MBs and dispersed in the assay system. After magnetic adsorption, the free AuNPs are collected and sent to ICP-MS for detection, while the AuNPs still attached to the MBs are left in the system [100,101]. ICP-MS signals not only determine the presence of HIV DNA, but also allow for its precise quantification. On the basis of Cas12a self-amplification, this method further amplifies the signal by enhancement effect of the large number of ^{197}Au inside each AuNP, thus enabling nucleic acid-free amplification detection of HIV DNA with a LOD of 1.05 amol in 40 min

3.2.3. SCAN

Another example of using nanomaterials combined with Cas12a to detect HIV DNA without nucleic acid amplification is the **so**lid-state **C**RISPR-Cas12a-**a**ssisted **n**anopores (SCAN) approach [102]. Unlike the use of nanomaterial AuNPs as reporter molecules and the introduction of expensive ICP-MS as signal detection instrument, the SCAN approach employs nanopores to generate signals and introduces a relatively inexpensive data acquisition device (6363 DAQ card) to develop a compact platform for accomplishing the acquisition and presentation of signals (Fig. 7). The nanopore sensor employed by SCAN is comprised of silicon nitride, glass, and graphene, which can sense the dip in current caused by the passage of the charged molecule through the orifice [103]. Since the extremely narrow orifice size of the solid-state nanopore allows the passage of only one charged molecule, a single dip in current means the translocation of one analyte, which makes nanopores capable of single-molecule detection [104]. However, a common challenge facing solid-state nanopores is maintaining a balance between specificity and versatility in sensing. Typically, nanopores have difficulty recognizing the species of the analyte without specific modifications, but the introduction of specific modifications in turn limits the potential of nanopores as general-purpose sensors and significantly increases the cost [105].

To address this problem, the SCAN approach coupled the CRISPR/Cas12a system with the solid-state nanopore. As the CRISPR/Cas12a system is capable of recognizing specific pathogen nucleic acid by synthetically engineered crRNA, the nanopore does not need to meet the requirement of recognizing the analyte but only needs to produce a consistent current signal to the analyte [102]. Specifically, when HIV DNA is detected by CRISPR/Cas12a, *trans*-cleavage of Cas12a is activated and thus the circular ssDNAs are cleaved and broken. Fewer intact cyclic ssDNAs pass through the solid-state nanopore, creating a lower electronic signal than when HIV DNA is not detected. Evaluation of the signal profile allows for the qualification and quantification of HIV DNA, however, SCAN has difficulty detecting HIV DNA below 10 nM as no nucleic acid amplification is conducted before [102]. Considering that the SCAN approach is compact and can be performed in situ, it is a suitable method for POC test of HIV DNA.

3.2.4. CRISPR-SERS-LFA

CRISPR-Cas12a mediated **s**urface **e**nhanced **R**aman **s**cattering **l**ateral **f**low **a**ssay (CRISPR-SERS-LFA) is also a method that relies on nanomaterials for nucleic acid amplification-free HIV DNA detection [106]. Importantly, most of the reactions of CRISPR-SERS-LFA can be integrated on a single lateral flow strip similar to the Cas9 nAR-v2, although the final signal collection requires specific equipment such as a Raman spectrometer (Fig. 7) [107].

The lateral flow strip of this method mainly contains the sample pad, the conjugate pad coupled with SiO2 @Au@DTNB-digoxin antibody, the test line with streptavidin, and the control line with anti-IgG [106]. In addition, the researchers designed a biotin-DNA-digoxin probe to act as a reporter molecule.

If the sample contains the target HIV DNA, the *cis*- and *trans*-activities of CRISPR/Cas12a are sequentially activated to cleave the biotin-DNA-digoxin probe, resulting in the biotin-DNA probe being captured by the streptavidin on the test line, emitting weak SERS signals; if the sample does not contain HIV DNA, the biotin-DNA-digoxin probe binds to SiO2 @Au@DTNB-digoxin antibody at the conjugate pad and then stays on the test line where the accumulated SiO2 @Au@DTNB emits intense SERS signals [107].

CRISPR-SERS-LFA can quantify HIV DNA with a LOD of 0.3 fM within 1 h, which is almost 4 orders of magnitude lower than that of traditional colorimetric LFA methods. Notably, the tool can identify HIV-1 single-base drug-resistant mutations (e.g., M184V) at a rate as low as 0.01%, demonstrating the high recognition specificity of Cas12a. It can be seen that CRISPR-SERS-LFA has great potential to become a POC detection method due to its ease of operation, high integration, and short time taken [106].

3.2.5. CRISPR-Cas12a-PCHA

Except for nanomaterials, combining with another method for detecting HIV DNA without nucleic acid amplification is called CRISPR-Cas12a-activated **p**alindrome **c**atalytic **h**airpin **a**ssembly (CRISPR-Cas12a-PCHA) [108]. The ingenuity of this approach lies in the design of four in vitro synthesized nucleic acid hairpins (H0, H1, H2, and H3), as well as their self-assembled formation of Y-shaped complexes to achieve fluorescence generation and amplification (Fig. 7) [109]. Specifically, H0 is a stem-loop formed by the hybridization of DNA to RNA and attached to MB. The H1, H2, and H3 have identical palindromic fragments at the 3′ end, meaning that they can undergo base complementary pairing with each other [108]. In addition, the stem end of H1 and H3 is labeled with FAM and BHQ, respectively.

The working principle of CRISPR-Cas12a-PCHA can be briefly described as following the CRISPR/Cas12a system recognizes the HIV DNA, the DNA fragment of the H1 is cleaved and removed by magnetic separation [108]. Then the released H0 RNA opens the stem of subsequently added H1 via a toehold-mediated hybridization reaction, which unblocks the fluorescence burst of FAM by BHQ. Similarly, H1 opens H2 with a free stem segment, and H2 in turn opens H3. Finally, base-pairing of H3 with the palindromic stem segment of H1 forms a Y-shaped H1/H2/H3 three-stranded complex and allows the RNA to be replaced and re-free, initiating the cyclic opening of H1 [108]. Since each individual RNA gets recycled, effective fluorescence signal amplification is realized to be detected by a fluorescence spectrophotometer. Despite avoiding upfront nucleic acid amplification, CRISPR-Cas12a-PCHA can still provide an ultrasensitive detection with a limit of 4.2 fM [108].

3.2.6. PEC-CRISPR/Cas12a assay

The method is a CRISPR/Cas12a-based assay employing hairpin nucleic acids and nanomaterials to detect HIV DNA and unique in that the recycling of the target HIV DNA and the catalytic product *p*-aminophenol (*p*-AP) form the basis for its elimination of the need for nucleic acid amplification [110].

Similar to CRISPR-Cas12a-PCHA, PEC-CRISPR/Cas12a assay utilizes a specially designed hairpin (H1) that initiates the entire reaction (Fig. 6). H1 is labeled with MB and can bind to HIV DNA to form the MB-H1-target complex that will be replaced by biotin-labeled H2 (biotin-H2) to form the H1-H2 complex and release the HIV DNA in order to

recycle it. The dsDNA formed by H1-H2 is designed to bind specifically to crRNA, which activates the *cis*-activity of Cas12a, leading to the separation of biotin-H2 and MB. If H1 fails to recognize HIV DNA, it cannot form the correct fold with H2 to activate CRISPR/Cas12a.

In addition, the researchers designed a PEC reaction. Through the addition of streptavidin-labeled alkaline phosphatase (SA-ALP), H2 and ALP are linked by the interaction of biotin and streptavidin. In the presence of HIV DNA, ALP escapes from the magnetic attraction with H2 by *trans*-activation of CRIPSR/Cas12a. Then ALP catalytic generates *p*-AP to produce quantifiable current signals through the (AuNPs)/MoO_2/Mo_2C-modified electrode [111]. Due to the existence of $Ru(bpy)_3^{2+}$, *p*-AP can be regenerated through redox reaction and thereby reused to amplify the signal, the method was able to accurately quantify 0.4 fM HIV DNA without nucleic acid amplification [112].

3.2.7. CRISPR-PGM platform

The self-amplification effect of Cas12a combined with enzyme catalysis also enables nucleic acid-free amplification of HIV DNA [113]. A study reported an improved CRISPR-personal glucometer readout (PGM) platform to detect HIV DNA by coupling Cas12a and invertase (Fig. 7) [113]. The PGM is a portable, commercially available glucose quantification instrument that has been investigated as a signaling transducer for biosensors. In order to obtain PGM readings, the detection of specific molecules is often linked to the release or capture of invertase or amylase.

In this research, the invertase is immobilized onto the MB by an ssDNA, i.e., the ssDNA-invertase conjugate is attached to the MB. Firstly, Cas12a cleaves ssDNA after *trans*-cleavage activation by HIV DNA, leading to the release of invertase. Then, after separation of free and MB-attached invertases using a magnetic frame, free invertases in the supernatant addition to millipore water containing sucrose, and eventual detection of generated glucose by commercial PGM, this platform successfully combines the self-amplification effect of Cas12a with enzyme-catalyzed amplification effect to detect HIV DNA for the POC test with the LOD of 11 fM within 80 min

3.2.8. Dual-QD-dCas9

CRISPR/Cas systems deliver not only qualitative and quantitative HIV DNA, but also imaging of HIV DNA within living cells [23]. A study has invented a quantum dot-based, two-color labelled CRISPR system to image HIV proviral DNA. QDs are semiconductor crystals of 2–10 nm in size that emit bright fluorescent light, the color of which can be changed by adjusting the size and the composition [114]. The researchers designed a dCas9 fused to a lipoic acid ligase (LplA) receptor peptide (LAP) and transfected it in the form of a plasmid, together with the LplA, the trans-cyclooctene (TCO2), and the tetrazine (Tz1)-QD625, into HIV-infected cells. In the presence of LplA, TCO2 is specifically ligated to LAP followed by a bio-orthogonal reaction between TCO2 and Tz1 to achieve QD625 labeling of dCas9 [115]. Guided by the sgRNA, CRISPR-dCas9-QD625 specifically targets HIV DNA without DNA destruction and instead provides real-time dynamic tracing and 3D localization analysis of the HIV provirus.

Considering that the fluorescence signal in the nucleus may be caused by random diffusion of the complex, which may interfere with the localization results. To overcome this problem, the researchers also utilized the similar idea to label dCas9 with QD525, which emits a different fluorescence than QD625 [23]. By observing the co-localization signal of QD625 and QD525 under fluorescence microscopy, accurate HIV provirus trace and determination of the number of integrated state proviruses within a single cell can be realized (Fig. 5). It is worth noting that one investigation used spherical nucleic acid labelled with carbon dots, a kind of nanomaterial that also has fluorescent properties, to detect HIV DNA in a "sandwich" concept similar to that of the ELISA assay. This sandwich approach combined with the CRISPR/Cas12a system allowed for the multiplex detection of HIV/HPV-16 DNAs, although CRISPR/Cas12a system was not directly used to detect HIV [116].

3.3. Both HIV DNA and RNA detection based on CRISPR/Cas system

The methods described above focus on the detection of HIV DNA or RNA, while some methods have been shown to detect both HIV DNA and RNA. These invented methods are achieved based on specific recognition of dsDNA by Cas12a and rely on nucleic acid amplification to provide a sufficient amount of substrate.

3.3.1. AIOD-CRISPR

An approach to HIV DNA and RNA detection based on Cas12a *trans*-activity plus RPA or RT-RPA is called the **a**ll-**i**n-**o**ne **d**ual CRISPR-Cas12a (AIOD-CRISPR) assay [117,118]. As the title suggests, AIOD-CRISPR assay is a one-pot system similar to rRT-RAA-Cas12a and CRISPR gel and is also characterized by simplicity, speed, and ultra-sensitivity [117].

Impressively, the AIOD-CRISPR assay further releases the detection potential of the CRISPR/Cas 12a system by simply adding crRNA species (Fig. 3). As a crRNA-dependent Cas12a complex occupies only one site in the target DNA, meaning that more sites conforming to the PAM restriction of Cas12a are still underutilized, the AIOD-CRISPR assay adopts a dual crRNAs-guided strategy to increase the amount of activated Cas12a and thus raise the signal [117]. Also, the amount of added ssDNA-FQ reporters is increased to reach the saturation level of the reaction system for full exploitation of the trans-cleavage of Cas12a, and the primer concentration is optimized. With these optimized operations, the AIOD-CRISPR assay exhibits a LOD of 1.2 HIV DNA copies and 11 HIV RNA copies per reaction (~20 μl) and fluoresces to the naked eye within 40 min by using a LED blue light illuminator [117]. This straightforward methodological optimization reaped comparable sensitivity with the quantitative PCR method and further demonstrated the strong potential of the CRISPR/Cas system for pathogen detection.

3.3.2. CRISPR-MCR

The CRISPR-**m**ediated **c**ascade **r**eaction (CRISPR-MCR) biosensor is an elaborate cascade reaction system that allows different reactions to take place in different spaces by means of compartments so that the reactions do not interfere with each other, which is inspired by the multi-compartmental, multi-enzymatic cascade reaction in eukaryotic cells [119].

Unlike the "one-pot" concept, the key to CRISPR-MCR is a screened and tested nanoporous polyether sulfone (PES) membrane that serves as the separator for separating the compartments (Fig. 4). The PES membrane separates the reaction system into an upper CRISPR chamber and a lower (RT)-RPA chamber, ensuring that the RPA reaction solution cannot interfere with the CRISPR reaction and detection. The reaction system is further integrated into a microfluidic platform that incorporates a blister pouch filled with water to act as a switch, and a PGM platform for electrochemical biosensing [120].

When HIV DNA/RNA samples were added into the (RT)-RPA chamber, a large number of short DNA amplicons were generated and diffused down the concentration gradient into the CRISPR chamber, thereby activating the *cis*- and *trans*-cleavage of the CRISPR/Cas12a system, causing the fracture of the ssDNA with the MB and invertase attached at the 5′-end and 3′-end, respectively (MB-ssDNA-invertase) [119]. The released invertase can no longer be magnetically adsorbed, but instead follows the water flow to the catalytic chamber to catalyze glucose production, which ultimately enables PGM detection. Using CRISPR-MCR, the researchers were able to consistently detect 43 copies of HIV DNA and 200 copies of HIV RNA per test within 5 min [119].

4. Conclusion and prospects

4.1. From POC to personal test

Several CRISPR-based assays have been developed that meet the criteria for POC testing. For instance, the Sherlock method-based kits were granted emergency use authorization by the Food and Drug Administration (FDA) for rapid diagnostics of SARS-CoV-2, thereby helping to alleviate the burden associated with virus testing [121]. Nevertheless, many current CRISPR-based methods detecting HIV still do not fully meet the requirements for POC testing. According to WHO standard clarifications and improvements based on the digital age, current POC tests should meet the REASSURED criteria: real-time connectivity, ease of specimen collection, affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free or simple, and deliverable to end-users [122]. Among the previously mentioned methods, CARMEN-Cas13, AuNPs-tagging-based CRISPR-Cas12a bioassay platform, and CRISPR-SERS-LFA involve high-value or technologically-threshold equipment, and the resulting increase in complexity of operation and analysis, causing a reduction in their user-friendliness and increasing the difficulty of delivering them to end-users. To this, modification and optimization toward "one-pot" or more integrated approach that used in detection for SARS-CoV-2 may be adapted for HIV detection [88,98,102,106,121].

One unmet need in HIV detection is the availability of early and accurate personal testing methods. Currently, personal HIV testing options mainly rely on antigen- and antibody-colloidal gold methods. Despite being simple and sensitive, these methods have a long window period and are thus limited in their effectivenes [123]. CRISPR-based methods, on the other hand, target specific HIV nucleic acids sequences and can effectively shorten the window period for HIV testing. Nonetheless, CRISPR-based methods still encounter many challenges in transitioning to a personal HIV detection method. For example, the current micro pipetting techniques are intricate and require specialized knowledge, which can be a barrier for non-experts. Furthermore, the use of many reagents and pipetting instruments can considerably increase the cost of the assay and make it less accessible to end-users. To address these issues, innovative approaches such as HUDSON (heating unextracted diagnostic samples to obliterate nucleases) may provide useful insights into simplifying the testing process [124]. HUDSON is a technique that eliminates the need for nucleic acid extraction by applying heat to inactivate nucleases. In summary, achieving a balance between maintaining high sensitivity and accuracy of the HIV nucleic acid assay and improving its user-friendliness and affordability is crucial to addressing the challenge at hand.

4.2. Nucleic acid amplification-free assay

The nucleic acid pre-amplification step leads to significant signal amplification and proves to be important in improving the sensitivity of the assay, but it may also amplify the impact of potential contamination. Currently, innovative methods for CRISPR-based HIV detection without nucleic acid amplification are costly. For instance, AuNPs-tagging based CRISPR-Cas12a bioassay platform requires ICP-MS for final detection and analysis. Similarly, SCAN requires nanopores to provide potential signals with a less optimal LOD [98,102]. Conversely, CRISPR-PGM and Cas12a-PCHA are favorable options with operational and cost benefits [108,113]. CRISPR-PGM only entails the addition of a conventional PGM, whereas Cas12a-PCHA is a self-assembled luminescent chemistry. Both of these methods offer good sensitivity and can eliminate the need for nucleic acid amplification while providing sufficient signals for observation. In fact, Cas12a-PCHA has been successfully utilized in clinical samples. The introduction of a third-party instrument or tool is a common and effective strategy, and in turn exploiting the characteristics of target objects themselves may also provide a good alternative. In light of the fact that a sufficiently long nucleic acid fragment provided multiple crRNA recognition sites, increasing the variety of crRNAs might improve the detection signal. An illustration of this was seen in AIOD-CRISPR, which employed dual-crRNA guidance for Cas12a cleavage and improved the fluorescence released by the ssDNA-FQ reporters.

4.3. Refined testing

Although qualitative and quantitative HIV testing is now possible with CRISPR-based assays, the complexity and variability of HIV demand further research on detecting multiple genotypes or drug-resistance mutations simultaneously. The rRT-RAA-Cas12a method can detect multiple HIV genotypes, which was achieved by rRT-RAA, meaning the potential of the Cas protein was not fully exploited [7]. In fact, the selection of the suitable Cas protein allows for the refined detection of the target nucleic acid. Cas13a has shown exquisite specificity in CARMEN-Cas13, detecting multiple drug-resistant strains of HIV, although the microwell array-based approach makes it more expensive [88]. Similarly, AacCas12b exhibited poor tolerance for mismatches between the guide RNA and the target dsDNA (even for single-base mismatch), which allows it to detect single-base mutations in cancer-related genes (TP53, BRCA1) for the purpose of highly refined detection [38]. Cas14a is another highly refined compact Cas protein that is less tolerant of mismatches near intermediate sequences, making it suitable for high-fidelity detection of SNPs [50]. These highly specific proteins (Cas12b, Cas13a, Cas14a, and other potential proteins), alone or in combination, have the potential to significantly improve HIV detection capabilities.

Contributions

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Declaration of Competing Interest

The authors declare no competing interest.

References

[1] https://www.un.org/zh/global-issues/aids.
[2] Yamazaki S, et al. Qualitative real-time PCR assay for HIV-1 and HIV-2 RNA. Jpn J Infect Dis 2016;69:367–72.
[3] Crooks AM, et al. Precise quantitation of the latent HIV-1 reservoir: implications for eradication strategies. J Infect Dis 2015;212:1361–5.
[4] Gibellini L, et al. Exploring viral reservoir: the combining approach of cell sorting and droplet digital PCR. Methods 2018;134–135:98–105.
[5] Lungu C, Procopio FA. TILDA: Tat/Rev induced limiting dilution assay. Methods Mol Biol 2022;2407:365–72.
[6] Freen-van Heeren JJ. Addressing HIV-1 latency with flow-FISH: finding, characterizing and targeting HIV-1 infected cells. Cytom A 2021;99:861–5.
[7] Zhao J, et al. A point-of-care rapid HIV-1 test using an isothermal recombinase-aided amplification and CRISPR Cas12a-mediated detection. Virus Res 2021;303:198505.
[8] Smith CJ, Osborn AM. Advantages and limitations of quantitative PCR (Q-PCR)-based approaches in microbial ecology. FEMS Microbiol Ecol 2009;67:6–20.
[9] Deveau H, Garneau JE, Moineau S. CRISPR/Cas system and its role in phage-bacteria interactions. Annu Rev Microbiol 2010;64:475–93.
[10] Mojica FJM, Díez-Villaseñor C, García-Martínez J, Almendros C. Short motif sequences determine the targets of the prokaryotic CRISPR defence system. Microbiology 2009;155:733–40.

[11] Srivastava S, Upadhyay DJ, Srivastava A. Next-generation molecular diagnostics development by CRISPR/Cas tool: rapid detection and surveillance of viral disease outbreaks. Front Mol Biosci 2020;7:582499.
[12] Bhardwaj P, Kant R, Behera SP, Dwivedi GR, Singh R. Next-generation diagnostic with CRISPR/Cas: beyond nucleic acid detection. Int J Mol Sci 2022;23:6052.
[13] Jinek M, et al. A programmable Dual-RNA–Guided DNA endonuclease in adaptive bacterial immunity. Science 2012;337:816–21.
[14] Ran FA, et al. Genome engineering using the CRISPR-Cas9 system. Nat Protoc 2013;8:2281–308.
[15] Nouri R, et al. CRISPR-based detection of SARS-CoV-2: a review from sample to result. Biosens Bioelectron 2021;178:113012.
[16] Selvam K, et al. RT-LAMP CRISPR-Cas12/13-Based SARS-CoV-2 detection methods. Diagnostics 2021;11.
[17] Makarova KS, Zhang F, Koonin EV. SnapShot: class 2 CRISPR-Cas systems. 328-328.e1 Cell 2017;168. 328-328.e1.
[18] Nishimasu H, et al. Crystal structure of Cas9 in complex with guide RNA and target DNA. Cell 2014;156:935–49.
[19] Jinek M, et al. Structures of Cas9 endonucleases reveal RNA-mediated conformational activation. Science 2014;343:1247997.
[20] Cong L, et al. Multiplex genome engineering using CRISPR/Cas systems. Science 2013;339:819–23.
[21] Kanafi MM, Tavallaei M. Overview of advances in CRISPR/deadCas9 technology and its applications in human diseases. Gene 2022;830:146518.
[22] Cebrian-Serrano A, Davies B. CRISPR-Cas orthologues and variants: optimizing the repertoire, specificity and delivery of genome engineering tools. Mamm Genome 2017;28:247–61.
[23] Ma Y, et al. Live visualization of HIV-1 proviral DNA using a dual-color-labeled CRISPR system. Anal Chem 2017;89:12896–901.
[24] Sander JD, Joung JK. CRISPR-Cas systems for editing, regulating and targeting genomes. Nat Biotechnol 2014;32:347–55.
[25] Ran FA, et al. Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. Cell 2013;154:1380–9.
[26] Yourik P, Fuchs RT, Mabuchi M, Curcuru JL, Robb GB. Staphylococcus aureus Cas9 is a multiple-turnover enzyme. RNA 2019;25:35–44.
[27] Ran FA, et al. In vivo genome editing using Staphylococcus aureus Cas9. Nature 2015;520:186–91.
[28] Dong D, et al. The crystal structure of Cpf1 in complex with CRISPR RNA. Nature 2016;532:522–6.
[29] Jiang F, Zhou K, Ma L, Gressel S, Doudna JA. Structural biology. A Cas9-guide RNA complex preorganized for target DNA recognition. Science 2015;348:1477–81.
[30] Zhang H, et al. Structural basis for the inhibition of CRISPR-Cas12a by anti-CRISPR proteins. e4 Cell Host Microbe 2019;25:815–26. e4.
[31] Chen JS, et al. CRISPR-Cas12a target binding unleashes indiscriminate single-stranded DNase activity. Science 2018;360:436–9.
[32] Stella S, Alcón P, Montoya G. Structure of the Cpf1 endonuclease R-loop complex after target DNA cleavage. Nature 2017;546:559–63.
[33] Swarts DC, Jinek M. Mechanistic Insights into the cis- and trans-Acting DNase activities of Cas12a. Mol Cell 2019;73(589–600):e4.
[34] Murugan K, Seetharam AS, Severin AJ, Sashital DG. CRISPR-Cas12a has widespread off-target and dsDNA-nicking effects. J Biol Chem 2020;295:5538–53.
[35] Teng F, et al. Repurposing CRISPR-Cas12b for mammalian genome engineering. Cell Discov 2018;4:63.
[36] Liu L, et al. C2c1-sgRNA complex structure reveals RNA-Guided DNA cleavage mechanism. Mol Cell 2017;65:310–22.
[37] Shmakov S, et al. Discovery and functional characterization of diverse class 2 CRISPR-Cas systems. Mol Cell 2015;60:385–97.
[38] Teng F, et al. CDetection: CRISPR-Cas12b-based DNA detection with sub-attomolar sensitivity and single-base specificity. Genome Biol 2019;20:132.
[39] Das A, Goswami HN, Whyms CT, Sridhara S, Li H. Structural principles of CRISPR-Cas enzymes used in nucleic acid detection. J Struct Biol 2022;214:107838.
[40] Liu L, et al. Two distant catalytic sites are responsible for C2c2 RNase activities. e12 Cell 2017;168:121–34. e12.
[41] Liu L, et al. The molecular architecture for RNA-Guided RNA cleavage by Cas13a. e10 Cell 2017;170:714–26. e10.
[42] Abudayyeh OO, et al. C2c2 is a single-component programmable RNA-guided RNA-targeting CRISPR effector. aaf5573 Science 2016;353. aaf5573.
[43] Abudayyeh OO, et al. RNA targeting with CRISPR-Cas13. Nature 2017;550:280–4.
[44] Zhang Z, et al. Capturing RNA-protein interaction via CRUIS. e52 Nucleic Acids Res 2020;48. e52.
[45] East-Seletsky A, et al. Two distinct RNase activities of CRISPR-C2c2 enable guide-RNA processing and RNA detection. Nature 2016;538:270–3.
[46] Slaymaker IM, et al. High-resolution structure of Cas13b and Biochemical Characterization of RNA Targeting and Cleavage. e5 Cell Rep 2019;26:3741–51. e5.
[47] Zhang B, et al. Structural insights into Cas13b-guided CRISPR RNA maturation and recognition. Cell Res 2018;28:1198–201.
[48] Brogan DJ, et al. Development of a rapid and sensitive CasRx-based diagnostic assay for SARS-CoV-2. ACS Sens 2021;6:3957–66.
[49] Zhang C, et al. Structural basis for the RNA-Guided ribonuclease activity of CRISPR-Cas13d. e17 Cell 2018;175:212–23. e17.
[50] Harrington LB, et al. Programmed DNA destruction by miniature CRISPR-Cas14 enzymes. Science 2018;362:839–42.
[51] Karvelis T, et al. PAM recognition by miniature CRISPR-Cas12f nucleases triggers programmable double-stranded DNA target cleavage. Nucleic Acids Res 2020;48:5016–23.
[52] Xiao R, Li Z, Wang S, Han R, Chang L. Structural basis for substrate recognition and cleavage by the dimerization-dependent CRISPR–Cas12f nuclease. Nucleic Acids Res 2021;49:4120–8.
[53] *Structure of the miniature type V-F CRISPR-Cas effector enzyme*. vol. 81 (2021).
[54] Luo ML, Mullis AS, Leenay RT, Beisel CL. Repurposing endogenous type I CRISPR-Cas systems for programmable gene repression. Nucleic Acids Res 2015;43:674–81.
[55] Caliando BJ, Voigt CA. Targeted DNA degradation using a CRISPR device stably carried in the host genome. Nat Commun 2015;6:6989.
[56] Makarova KS, et al. An updated evolutionary classification of CRISPR-Cas systems. Nat Rev Microbiol 2015;13:722–36.
[57] Kim SK, et al. CRISPRi-based programmable logic inverter cascade for antibiotic-free selection and maintenance of multiple plasmids. Nucleic Acids Res 2022;50:13155–71.
[58] Dolan AE, et al. Introducing a spectrum of long-range genomic deletions in human embryonic stem cells using Type I CRISPR-Cas. e5 Mol Cell 2019;74:936–50. e5.
[59] Brouns SJJ, et al. Small CRISPR RNAs guide antiviral defense in prokaryotes. Science 2008;321:960–4.
[60] Shangguan Q, Graham S, Sundaramoorthy R, White MF. Structure and mechanism of the type I-G CRISPR effector. Nucleic Acids Res 2022;50:11214–28.
[61] Shahbazi R, Lipson P, Gottimukkala KSV, Lane DD, Adair JE. CRISPR Gene Editing of Hematopoietic Stem and Progenitor Cells. Methods Mol Biol 2023;2567:39–62.
[62] Kazlauskiene M, Kostiuk G, Venclovas Č, Tamulaitis G, Siksnys V. A cyclic oligonucleotide signaling pathway in type III CRISPR-Cas systems. Science 2017;357:605–9.
[63] Zhu X, Ye K. Crystal structure of Cmr2 suggests a nucleotide cyclase-related enzyme in type III CRISPR-Cas systems. FEBS Lett 2012;586:939–45.
[64] Amitai G, Sorek R. Intracellular signaling in CRISPR-Cas defense. Science 2017;357:550–1.
[65] Cocozaki AI, et al. Structure of the Cmr2 subunit of the CRISPR-Cas RNA silencing complex. Structure 2012;20:545–53.
[66] Elmore JR, et al. Bipartite recognition of target RNAs activates DNA cleavage by the Type III-B CRISPR-Cas system. Genes Dev 2016;30:447–59.
[67] Niewoehner O, et al. Type III CRISPR-Cas systems produce cyclic oligoadenylate second messengers. Nature 2017;548:543–8.
[68] Burroughs AM, Zhang D, Schäffer DE, Iyer LM, Aravind L. Comparative genomic analyses reveal a vast, novel network of nucleotide-centric systems in biological conflicts, immunity and signaling. Nucleic Acids Res 2015;43:10633–54.
[69] Niewoehner O, Jinek M. Structural basis for the endoribonuclease activity of the type III-A CRISPR-associated protein Csm6. RNA 2016;22:318–29.
[70] You L, et al. Structure studies of the CRISPR-Csm complex reveal mechanism of Co-transcriptional Interference. e16 Cell 2019;176:239–53. e16.
[71] Sridhara S, Goswami HN, Whyms C, Dennis JH, Li H. Virus detection via programmable Type III-A CRISPR-Cas systems. Nat Commun 2021;12:5653.
[72] Zhou Y, et al. Structure of a type IV CRISPR-Cas ribonucleoprotein complex. iScience 2021;24:102201.
[73] Hiv TL. U⁀U taking off in 2017. Lancet HIV 2017;4:e475.
[74] Cohen MS, et al. Antiretroviral therapy for the prevention of HIV-1 transmission. New Engl J Med 2016;375:830–9.
[75] Rodger AJ, et al. Risk of HIV transmission through condomless sex in serodifferent gay couples with the HIV-positive partner taking suppressive antiretroviral therapy (PARTNER): final results of a multicentre, prospective, observational study. Lancet 2019;393:2428–38.
[76] Wang T, Wang Y, Chen P, Yin B-C, Ye B-C. An ultrasensitive, One-Pot RNA detection method based on rationally engineered Cas9 nickase-assisted isothermal amplification reaction. Anal Chem 2022;94:12461–71.
[77] Walker GT, et al. Strand displacement amplification–an isothermal, in vitro DNA amplification technique. Nucleic Acids Res 1992;20:1691–6.
[78] Richardson CD, Ray GJ, DeWitt MA, Curie GL, Corn JE. Enhancing homology-directed genome editing by catalytically active and inactive CRISPR-Cas9 using asymmetric donor DNA. Nat Biotechnol 2016;34:339–44.
[79] Kleinstiver BP, et al. High-fidelity CRISPR-Cas9 nucleases with no detectable genome-wide off-target effects. Nature 2016;529:490–5.
[80] Chen JS, et al. Enhanced proofreading governs CRISPR-Cas9 targeting accuracy. Nature 2017;550:407–10.
[81] Slaymaker IM, et al. Rationally engineered Cas9 nucleases with improved specificity. Science 2016;351:84–8.
[82] Shen X-X, et al. A rapid and sensitive recombinase aided amplification assay to detect hepatitis B virus without DNA extraction. BMC Infect Dis 2019;19:229.
[83] Fan X, et al. Clinical validation of two recombinase-based isothermal amplification assays (RPA/RAA) for the rapid detection of African swine fever virus. Front Microbiol 2020;11:1696.
[84] Uno N, Li Z, Avery L, Sfeir MM, Liu C. CRISPR gel: a one-pot biosensing platform for rapid and sensitive detection of HIV viral RNA. Anal Chim Acta 2023;1262:341258.
[85] Hoan TNgo, et al. Rapid and portable quantification of HIV RNA via a smartphone-enabled digital CRISPR device and deep learning. 2023.05.12.23289911 medRxiv 2023. https://doi.org/10.1101/2023.05.12.23289911.
[86] Ngo HL, Nguyen HD, Tran VN, Ngo HT. Development of a low-cost and portable real-time PCR machine for developing countries. In: Van Toi V, Nguyen T-H,

Long VB, Huong HTT, editors. 8th International Conference on the Development of Biomedical Engineering in Vietnam. Springer International Publishing; 2022. p. 69–83.
[87] F.Ö. Ünel B.O. Özkalayci C. Çiğla The Power of Tiling for Small Object Detection 2019 IEEE/CVF Conf Comput Vis Pattern Recognit Workshops (CVPRW) 2019 582 591 doi: 10.1109/CVPRW.2019.00084.
[88] Ackerman CM, et al. Massively multiplexed nucleic acid detection with Cas13. Nature 2020;582:277–82.
[89] Storch GA. CRISPR tool scales up to interrogate a huge line-up of viral suspects. Nature 2020;582:188–9.
[90] Gootenberg JS, et al. Nucleic acid detection with CRISPR-Cas13a/C2c2. Science 2017;356:438–42.
[91] Nouri R, et al. STAMP-based digital CRISPR-Cas13a for amplification-free quantification of HIV-1 plasma viral loads. ACS Nano 2023;17:10701–12.
[92] Huang X, et al. Smartphone-Based in-Gel Loop-Mediated Isothermal Amplification (gLAMP) System Enables Rapid Coliphage MS2 Quantification in Environmental Waters. Environ Sci Technol 2018;52:6399–407.
[93] J. Ortega, M. Rocío B. Rojas M. García Research issues on K-means algorithm: an experimental trial using matlab CEUR Workshop Proc 534 2009.
[94] Qin L, et al. CRISPR/Cas12a coupled with enzyme-DNA molecular switch photoelectrochemical assay for HIV nucleic acid. Microchem J 2023;190:108713.
[95] Lin Y, Jayasena SD. Inhibition of multiple thermostable DNA polymerases by a heterodimeric aptamer. J Mol Biol 1997;271:100–11.
[96] Jung Y, Lee CY, Park KS, Park HG. Target-activated DNA polymerase activity for sensitive RNase H activity assay. e1800645 Biotechnol J 2019;14. e1800645.
[97] Liu T, Sun D, Gu M, Wu X, Wang G-L. Intercalative methylene blue as an efficient signal molecule of tremella-like Bi2WO6: toward high performance photoelectrochemical biosensing. Sens Actuators B: Chem 2020;317:128210.
[98] Zhou J, Hu J, Liu R, Wang C, Lv Y. Dual-amplified CRISPR-Cas12a bioassay for HIV-related nucleic acids. Chem Commun 2022;58:4247–50.
[99] Viswambari Devi R, Doble M, Verma RS. Nanomaterials for early detection of cancer biomarker with special emphasis on gold nanoparticles in immunoassays/sensors. Biosens Bioelectron 2015;68:688–98.
[100] Zhang C, Zhang Z, Yu B, Shi J, Zhang X. Application of the biological conjugate between antibody and colloid Au nanoparticles as analyte to inductively coupled plasma mass spectrometry. Anal Chem 2002;74:96–9.
[101] Liu R, et al. Metal stable isotope tagging: renaissance of radioimmunoassay for multiplex and absolute quantification of biomolecules. Acc Chem Res 2016;49:775–83.
[102] Nouri R, Jiang Y, Lian XL, Guan W. Sequence-specific recognition of HIV-1 DNA with solid-state CRISPR-Cas12a-assisted nanopores (SCAN). ACS Sens 2020;5:1273–80.
[103] Firnkes M, Pedone D, Knezevic J, Döblinger M, Rant U. Electrically facilitated translocations of proteins through silicon nitride nanopores: conjoint and competitive action of diffusion, electrophoresis, and electroosmosis. Nano Lett 2010;10:2162–7.
[104] Miles BN, et al. Single molecule sensing with solid-state nanopores: novel materials, methods, and applications. Chem Soc Rev 2013;42:15–28.
[105] Keyser UF. Enhancing nanopore sensing with DNA nanotechnology. Nat Nanotechnol 2016;11:106–8.
[106] Pang Y, et al. CRISPR-cas12a mediated SERS lateral flow assay for amplification-free detection of double-stranded DNA and single-base mutation. Chem Eng J 2022;429:132109.
[107] Wang L, et al. SERS-based test strips: Principles, designs and applications. Biosens Bioelectron 2021;189:113360.
[108] Zhao, X. CRISPR-Cas12a-activated palindrome-catalytic hairpin assembly for ultrasensitive fluorescence detection of HIV-1 DNA. *Analytica Chimica Acta.*
[109] Wu N, et al. Three-dimensional DNA nanomachine biosensor by integrating DNA walker and rolling machine cascade amplification for ultrasensitive detection of cancer-related gene. Anal Chem 2020;92:11111–8.
[110] Qin N, Deng L, Wang M, Hun X. Gold nanoparticles/Mo2C/MoO2-Modified Electrodes for Nucleic Acid Detection through CRISPR/Cas12a Photoelectrochemical Assay. ACS Appl Nano Mater 2021;4:10701–7.
[111] Eames C, Islam MS. Ion intercalation into two-dimensional transition-metal carbides: global screening for new high-capacity battery materials. J Am Chem Soc 2014;136:16270–6.
[112] Hun X, Meng Y. Electron acceptors co-regulated self-powered photoelectrochemical strategy and its application for circulating tumor nucleic acid detection coupled with recombinase polymerase amplification. Anal Chem 2020;92:11771–8.
[113] Fang B, et al. A versatile CRISPR Cas12a-based point-of-care biosensor enabling convenient glucometer readout for ultrasensitive detection of pathogen nucleic acids. Talanta 2022;249:123657.
[114] Le N, Zhang M, Kim K. Quantum dots and their interaction with biological systems. Int J Mol Sci 2022;23.
[115] Liu DS, et al. Diels-Alder cycloaddition for fluorophore targeting to specific proteins inside living cells. J Am Chem Soc 2012;134:792–5.
[116] Zhao K-R, et al. A signal-switchable electrochemiluminescence biosensor based on the integration of spherical nucleic acid and CRISPR/Cas12a for multiplex detection of HIV/HPV DNAs. Sens Actuators B: Chem 2021;346:130485.
[117] Ding X, Yin K, Li Z, Liu C. All-in-one dual CRISPR-Cas12a (AIOD-CRISPR) assay: a case for rapid, ultrasensitive and visual detection of novel coronavirus SARS-CoV-2 and HIV virus. 2020.03.19.998724 Preprint at bioRxiv: Prepr Serv Biol 2020. https://doi.org/10.1101/2020.03.19.998724. 2020.03.19.998724 Preprint at.
[118] Ding X, et al. Ultrasensitive and visual detection of SARS-CoV-2 using all-in-one dual CRISPR-Cas12a assay. Nat Commun 2020;11:4711.
[119] Li Z, et al. Bioinspired CRISPR-mediated cascade reaction biosensor for molecular detection of HIV using a glucose meter. ACS Nano 2023;17:3966–75.
[120] Orooji Y, et al. Nanostructured mesoporous carbon polyethersulfone composite ultrafiltration membrane with significantly low protein adsorption and bacterial adhesion. Carbon 2017;111:689–704.
[121] Guglielmi G. First CRISPR test for the coronavirus approved in the United States. Nature 2020. https://doi.org/10.1038/d41586-020-01402-9.
[122] Otoo JA, Schlappi TS. REASSURED multiplex diagnostics: a critical review and forecast. Biosensors 2022;12.
[123] Guillon G, Yearwood G, Snipes C, Boschi D, Reed MR. Human anti-HIV IgM detection by the OraQuick ADVANCE® Rapid HIV 1/2 antibody test. PeerJ 2018;6:e4430.
[124] Myhrvold C, et al. Field-deployable viral diagnostics using CRISPR-Cas13. Science 2018;360:444–8.