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BIOSENSING PLATFORMS FOR DNA DIAGNOSTICS BASED ON CRISPR/Cas NUCLEASES: TOWARDS THE DETECTION OF NUCLEIC ACIDS AT THE LEVEL OF SINGLE MOLECULES IN NON-LABORATORY SETTINGS

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The use of CRISPR/Cas nucleases for the development of DNA diagnostic systems in out-of-laboratory conditions (point-of-need testing, PONT) has demonstrated rapid growth in the last few years, starting with the appearance in 2017–2018 of the first diagnostic platforms known as DETECTR and SHERLOCK. The platforms are based on a combination of methods of nucleic acid isothermal amplification with selective CRISPR/Cas detection of target amplicons. This significantly improves the sensitivity and specificity of PONT, making them comparable with or even superior to the sensitivity and specificity of polymerase chain reaction, considered as the “gold standard” of DNA diagnostics. The review considers modern approaches to the coupling of CRISPR/Cas detection using Cas9, Cas12a, Cas12b, Cas13a, Cas14, and Cas3 nucleases to various methods of nucleic acid isothermal amplification, with an emphasis on works in which sensitivity at the level of single molecules (attomolar and subattomolar concentrations of the target) is achieved. The properties of CRISPR/Cas nucleases used for targeted DNA diagnostics and the features of methods of nucleic acid isothermal amplification are briefly considered in the context of the development of diagnostic biosensing platforms. Special attention is paid to the most promising directions for the development of DNA diagnostics using CRISPR/Cas nuclease.

Key words: CRISPR/Cas nucleases; nucleic acid detection; single molecules; DNA diagnostics

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INTRODUCTION

The invention of polymerase chain reaction (PCR) by Kary Mullis in 1983 led to the emergence of a new area of research and practical applications — DNA diagnostics. DNA diagnostics is now broadly used in practical medicine and biomedical studies to identify disorders in genes that determine hereditary diseases and to detect pathogens causing infectious diseases [1]. Based on the enzymatic amplification of a specific section of genome or cDNA (complementary DNA synthesized *in vitro* by reverse transcriptase on RNA as a template) as a result of controlled multiple cyclic alterations in the temperature of the reaction mixture, PCR is the “gold standard” of DNA diagnostics. However, PCR requires complex expensive equipment (amplifiers/thermocyclers) that limits the use of this method to specialized

laboratories [2]. Although mobile PCR systems for out-of-laboratory diagnostics are currently being developed (see for example, [3]), they still remain complex and expensive equipment.

Along with PCR, today there are methods of nucleic acid amplification carried out at a constant temperature [4, 5], the use of which can significantly reduce the technical complexity of the required equipment and, consequently, the overall cost of testing. Such methods are grouped under a common name “isothermal amplification” and considered as the most promising approach to the practical implementation of DNA diagnostics in the format of “point-of-care testing” (POST) [6] or, in a broader context, in the format of out-of-laboratory DNA diagnostics (“point-of-need testing”, PONT). The trend towards the development of biosensing systems for such

Abbreviations used: CRISPR – clustered regularly interspaced short palindromic repeats; Cas – CRISPR associated protein; DFHBI – 3,5-difluoro-4-hydroxybenzylidene imidazolinone; cDNA – complementary DNA; ssDNA – single-stranded DNA; dsDNA – double-stranded DNA; EXPAR – exponential amplification reaction; FAM – 6-carboxyfluorescein; HCG – human chorionic gonadotropin; HPV – human papillomavirus; LAMP – loop-mediated isothermal amplification; LFA – Lateral Flow Assay; LOD – limit of detection; NASBA – nucleic acid sequence based amplification; PAM – protospacer adjacent motif; PCR – polymerase chain reaction; POCT – point-of-care testing; PONT – point-of-need testing; RCA – rolling circle amplification; gRNA – guide RNA; crRNA – CRISPR-RNA; tracrRNA – *trans*-activating RNA; sgRNA – single guide RNA; RPA – recombinase polymerase amplification; RT-RPA – reverse transcription-RPA; SDA – strand displacement amplification; ROX – carboxy-X-rhodamine.

testing has taken shape in the last two decades, having recently received an additional impact as a result of the SARS-CoV-2 pandemic [7, 8] and the growing awareness of problems related to the biosafety of population in the modern world [9, 10].

Compared with PCR, isothermal amplification is characterized by generally lower selectivity [11]. This is largely due to the longer length and compositional complexity of primers used that leads to a high probability of primer dimer formation and makes the design and experimental verification of primers an extremely time-consuming task without guaranteed success. In practice, the lower selectivity can lead to false positive test results due to the possibility of generating “off-target” amplicons [11]. Since 2016, CRISPR/Cas nucleases (CRISPR, clustered regularly interspaced short palindromic repeats; Cas, CRISPR associated protein), widely known as a successful toolkit for genomic editing [12] (its development was awarded the 2020 Nobel Prize in Chemistry [13]), have been in focus as probably the most effective solution to the selectivity problem associated with isothermal amplification. The coupling of CRISPR/Cas nucleases with isothermal amplification made it possible to create a new approach to the ultra-sensitive and highly selective detection of nucleic acids (primarily DNA and RNA molecules of various microorganisms and viruses [14]), providing the ultimate detection sensitivity of the biosensing platforms, up to the level of attomolar and subattomolar target concentrations [14]. To date, several diagnostic platforms have been proposed using CRISPR/Cas nucleases and various isothermal amplification methods: SHERLOCK [15], DETECTR [16], HOLMES [17], HUDSON [18], CARMEN [19], and a number of others, as well as their various modifications.

The review surveys the current development of biosensing systems for PONT, where CRISPR/Cas nucleases Cas9, Cas12a and Cas12b, Cas13a, Cas14 (another name Cas12f), and Cas3 are employed, with an emphasis on studies aimed at achieving detection sensitivity at the level of single molecules. A brief description of these CRISPR/Cas nucleases and isothermal amplification methods used in conjunction with them is also given, and the most promising directions for the development of biosensing systems based on CRISPR/Cas nucleases are discussed.

1. CLASSIFICATION AND CHARACTERIZATION OF Cas PROTEINS USED IN THE DETECTION OF NUCLEIC ACIDS

In nature, CRISPR/Cas nucleases are involved in the immune response of bacteria and archaea to phages, viruses and other foreign genetic elements invading the cell [20]. When they first enter the cell, a section of the foreign gene is integrated

into the genome of the host microorganism with the participation of effector proteins Cas1, Cas2, and a number of others, becoming part of the set of repeating sequences in the microorganism genome, known as CRISPR. During their transcription, specific RNA molecules are produced by ribonuclease III. These RNAs form complexes with Cas nucleases, determining the recognition of foreign sequences by the complex that leads to the activation of nuclease and cleavage of foreign DNA. For a number of Cas nucleases, the formation of the complex occurs with a single RNA molecule (guide RNA; gRNA), while for others two RNA molecules are needed: one determines the interaction with the target (CRISPR-RNA; crRNA), and another with Cas nuclease (*trans*-activating RNA; tracrRNA) [20]. In artificial systems, these two RNA molecules are usually united into one single guide RNA (sgRNA), which in the frame of this review will also be called “guide RNA” (gRNA) for simplicity. The sequence of gRNA consists of two parts — one is responsible for the formation of a complex with Cas nuclease (repeat), and the sequence of another (spacer) is complementary to sequence in the target (protospacer). Diagnostic platforms use recombinant CRISPR/Cas nucleases obtained by their heterologous expression followed by three- or two-stage chromatographic purification (e.g., [16, 21]), although examples of successful use for DNA diagnostics of recombinant CRISPR/Cas nucleases obtained by single-stage chromatographic purification are also known (e.g., [22, 23]).

Currently, the most accepted classification of Cas nucleases is proposed in [24], where they are divided into two classes, 1 and 2, primarily based on the structure of effector complexes, which include, respectively, either several Cas proteins (Class 1) or a single multi-domain Cas protein (Class 2). Each class is divided into types: Class 1 — types I, III, and IV, differing in the composition of Cas proteins in the effector complex, and Class 2 — types II, V, and VI, differing in the structural organization of Cas effector, the structure of gRNA and the nature of the target [24]. With the exception of Cas3, all Cas nucleases considered in this review (Cas9, Cas12, Cas13) belong to Class 2, that is, they enter the effector complex with gRNA as a single multidomain protein.

CRISPR/Cas9 consists of three domains: one recognizing gRNA and two others exhibiting enzymatic activity (HNH and RuvC domains) [25]. When forming a complex with gRNA, Cas9 nuclease is able to recognize the target sequence in double-stranded DNA (dsDNA) and make nicks in each of the strands in certain positions (Fig. 1A). Interestingly, when using gRNA as a single molecule, Cas9 exhibits only *cis*-activity — the nuclease activity against the target (in this case, the DNA target). However, as recently shown for the Cas9 nuclease originating from *Streptococcus pyogenes* (SpyCas9),

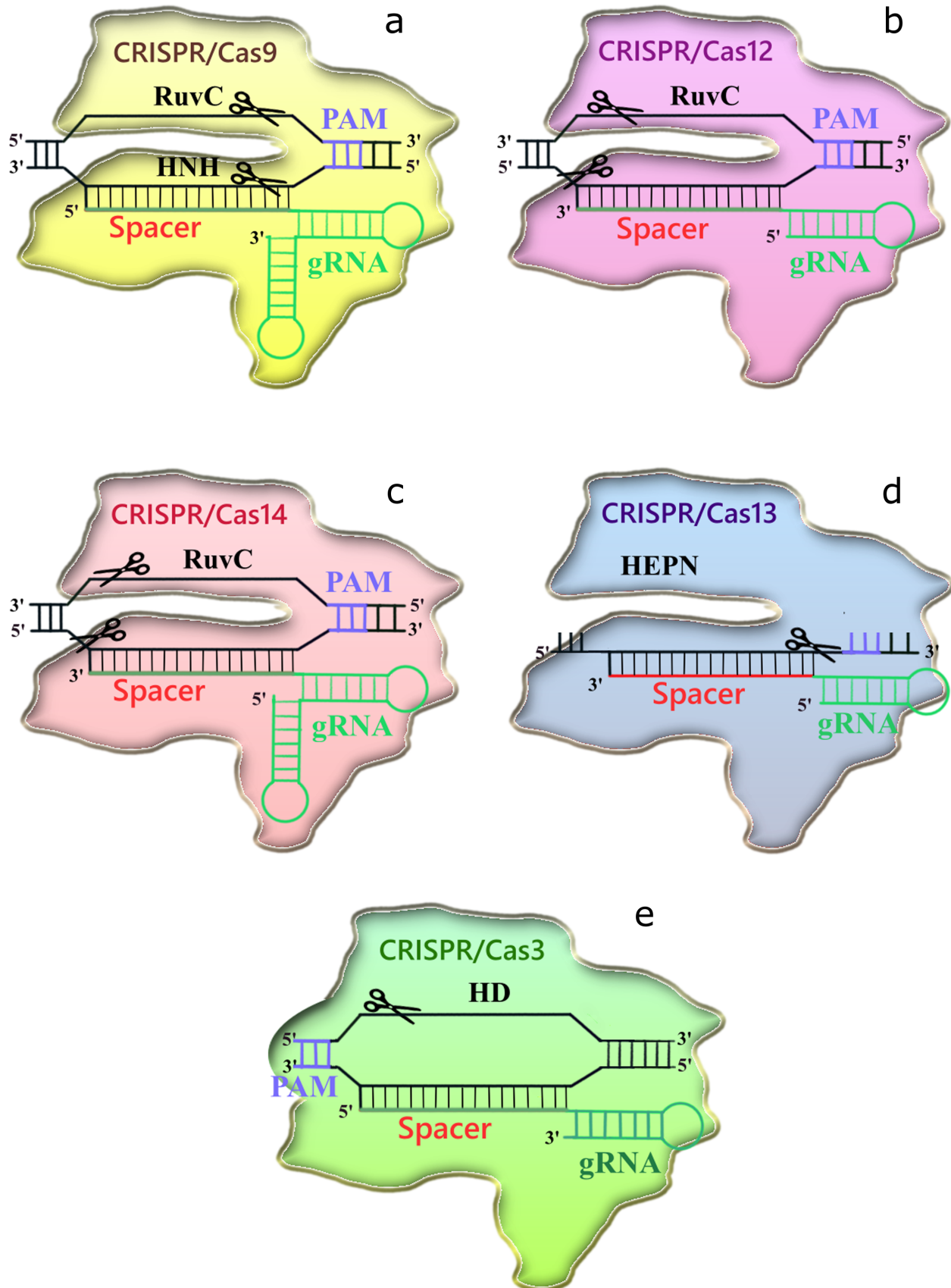


Figure 1. Schematic illustration of the DNA or RNA target recognition by CRISPR/Cas nucleases Cas9 (a), Cas12 (b), Cas14 (c), Cas13 (d), and Cas3 (e).

the use of crRNA and tracrRNA in an artificial system leads to acquiring *trans*-activity (or collateral activity) by the nuclease — the ability to non-specifically cleave DNA and RNA oligonucleotides (in this case, representing either polythymidines or polyadenines) after the “recognition” of the protospacer sequence in the target DNA by the crRNA spacer (the formation of the protospacer/spacer duplex) [26]. An important factor necessary for the activation of Cas9 is the presence of a specific motif from the 3'-end in the target sequence complementary to the protospacer — the so-called “protospacer adjacent motif” (PAM, Fig. 1A). As a rule, this is the three-nucleotide motif NGG [25], but Cas9 nucleases with alternative PAMs are also known [27]. Moreover, a number of recombinant SpyCas9 mutants have been obtained to date, which have significantly reduced PAM requirements — the presence of the NRNH sequence (R = A/G, H = A/C/T) is sufficient for their activation [28, 29].

CRISPR/Cas12a and CRISPR/Cas12b nucleases (also known as Cpf1 and C2c1, respectively) have a single domain with nuclease activity, RuvC, and, in the complex with gRNA, recognize the protospacer sequence in dsDNA, making a nick in each dsDNA strands [30]. The recognition requires the presence of a thymine-rich PAM (TTTV, where V = A/C/G) in the target dsDNA at the 5'-end of a sequence complementary to the protospacer (Fig. 1B). In addition, orthologs of Cas12a with other PAMs are known: RR and RVR [31]. In cells, CRISPR/Cas12a interacts with a sgRNA, whereas the CRISPR/Cas12b nuclease requires crRNA and tracrRNA. But in artificial systems, in both cases, gRNA is used. Both nucleases are also able to recognize the protospacer sequence in single-stranded DNA (ssDNA). After activation, they acquire *trans*-activity against ssDNA [30]. Interestingly, as has been shown recently, Cas12a can also be *trans*-activated by the presence of a target RNA [32].

CRISPR/Cas14 nuclease (now re-classified as Cas12f [33]) has a domain organization and properties similar to other CRISPR/Cas12 nucleases (including the requirement for the presence of thymine-rich PAM [34], Fig. 1B), but differs in size: CRISPR/Cas14 nucleases obtained from different organisms contain 400–700 amino acid (aa) residues in contrast to 950–1400 aa residues for Cas9 and Cas12 [35].

In contrast to the CRISPR/Cas nucleases described above, Cas13a in the complex with gRNA recognizes the protospacer in the target RNA sequence [36]. CRISPR/Cas13a has two domains with ribonuclease activity, HEPN-1 and HEPN-2, and after activation (no presence of PAM is required for that) acquires collateral activity, consisting in the ability to non-specifically cleave RNA molecules (Fig. 1G) [36].

Unlike the Cas nucleases discussed above, Cas3 belongs to Class 1 CRISPR/Cas nucleases, being a common part of the multi-protein CRISPR/Cas systems.

Cas3 consists of two domains, SF3 and HD, with helicase and endonuclease activities, respectively [37]. The activity of Cas3 depends on the presence of ATP and is aimed at cleavage of a non-complementary sequence in the target DNA in the presence of PAM at the 5'-end (Fig. 1D). The collateral activity of Cas3 leads to the cleavage of ssDNA molecules [37].

2. ISOTHERMAL AMPLIFICATION METHODS USED IN COMBINATION WITH CRISPR/Cas DETECTION OF TARGET AMPLICONS

2.1. Loop-Mediated Isothermal Amplification

Loop-mediated isothermal amplification (LAMP) is the most broadly used method of isothermal amplification of DNA and cDNA. Proposed in 2000 by T. Notomi [38], the method is based on the use of a polymerase with strand displacing activity and two or three pairs of primers, in one of which the primers have a specific composition. Two pairs of primers, “inner” and “outer”, employed in the original LAMP version [38], make it possible to start the reaction in the format of “nested amplification” (Fig. 2A) — the elongation of the “outer” primers by polymerase leads to a displacement of the newly synthesized chain originating at the 3'-end of the “inner” primer and the formation of a “dumbbell-shaped” structure, which is further exponentially amplified due to the interaction of the “inner” primers and loop-shaped sections of the formed amplicons. The formation of a dumbbell-like structure occurs due to a certain design of the “inner” primers: one part of their sequence (at the 5'-end) is complementary to a section of the sequence in the DNA target chain, while the sequence of the second part (at the 3'-end) is identical to a section of the sequence in another DNA target chain (Fig. 2A). A third pair of the primers, the loop primers, was proposed later to accelerate the amplification reaction [39]. LAMP is carried out at a temperature of 60–65°C and provides at least a 10⁹-fold increase in the number of copies of the target DNA in 0.5-1 hour [5, 38, 39].

2.2. Recombinase Polymerase Amplification

Recombinase polymerase amplification (RPA) was proposed in 2006 [40] and is based on the ability of phage T4 UvsX recombinase to form complexes with ssDNA — in this case, with primers — and to embed them in the presence of AMP into template dsDNA at a site with a sequence complementary to the primer. The resulting structure is stabilized by the gp32 protein of the T4 phage, which has an affinity for ssDNA (Fig. 2B). After that Bsu polymerase (with strand displacing activity) synthesizes a new DNA chain, starting from the 3'-end of the primer. RPA is conducted at 37–42°C and provides a 10⁹-fold increase in the number of copies of the target DNA within 20–40 minutes [5, 40].

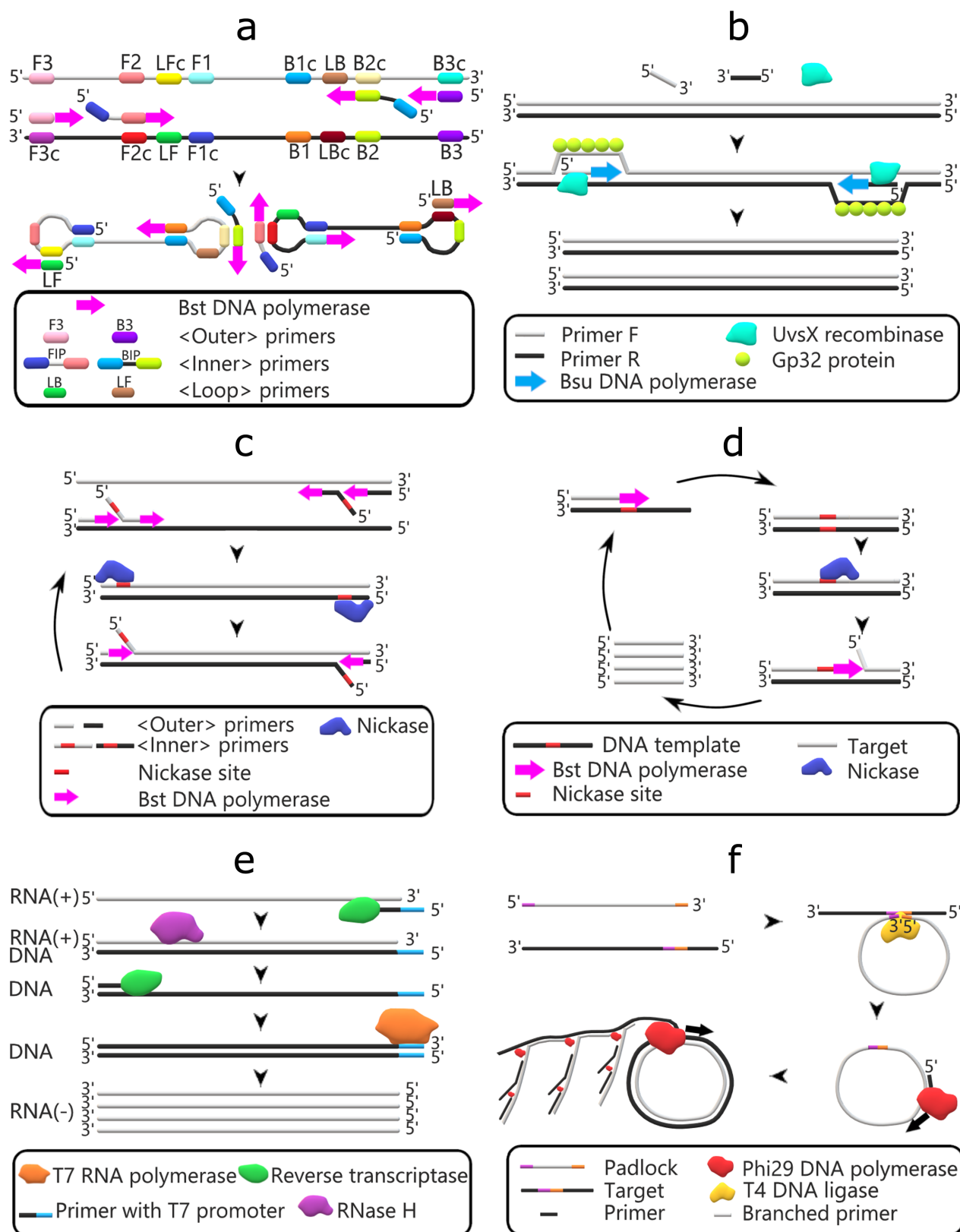


Figure 2. Schematic illustration of isothermal amplification methods. **a:** Loop-mediated isothermal amplification (LAMP). The annealing sites of the “outer”, “inner”, and loop primers on the strands of target DNA are shown, the extension “c” indicates the complementarity of sequences. **b:** Recombinase polymerase amplification (RPA). **c:** Strand displacing amplification (SDA). **d:** Exponential amplification reaction (EXPAR). **e:** Nucleic acid sequence based amplification (NASBA). RNA(+) represents the target RNA (sense RNA), RNA(-) is a complementary RNA (antisense RNA) obtained as a result of amplification. **f:** Rolling circle amplification (RCA).

2.3. Strand Displacement Amplification

Strand displacement amplification (SDA), proposed more than three decades ago [41], is based on the synthesis of a new strand by polymerase with strand displacing activity from the 3'-end of an external primer (bump primer), resulting in displacement of the newly synthesized chain, which originates at the 3'-end of the internal primer. The internal primer contains the additional sequence non-complementary to the DNA sequence of the target, which has a restriction site (Fig. 2B). Once the new template with the restriction site appears, it begins to be recognized by a nickase, making a nick in the DNA strand and thus triggering the exponential amplification of the target. Originally, SDA was carried out at 37°C, with the initial incubation of a mixture of primers and a target at 95°C [41], however, after the appearance of thermophilic enzymes, SDA has started to be carried out at a constant temperature in the range of 55–65°C, leading to a 10¹⁰-fold increase in the number of copies of the target DNA [5, 41].

2.4. Exponential Amplification Reaction

Exponential amplification reaction (EXPAR) was developed in 2003 [42] and, like SDA, is based on the combined action of a nickase and polymerase with strand displacing activity. The reaction can take place with both linear and exponential amplification of the target DNA at 60°C, providing in the latter case a 10⁶-fold increase in the number of copies of a short section of the target DNA (signal sequence) [42]. EXPAR uses a specific ssDNA template consisting of two repeating sequences, complementary to the signal sequence, between which the nickase recognition site is located. As a result of the reaction, short cDNA fragments identical to the signal sequence are synthesized (Fig. 2G).

2.5. Nucleic Acid Sequence Based Amplification

Nucleic acid sequence based amplification (NASBA) allows for direct RNA amplification: RNA molecules are used as targets and they are also amplicons generated as a result of the reaction (however, the sequence of RNA amplicons is complementary to the sequence of the target RNA) [43]. Amplification is achieved by the combined action of a combination of enzymes — reverse transcriptase, RNase H, and T7 RNA polymerase, while one of the primers contains an additional sequence representing the promoter of T7 RNA polymerase (Fig. 2D). The amplification of RNA molecules is mediated by the formation of an intermediate product — a dsDNA template containing the T7 RNA polymerase promoter. The reaction is carried out at 41°C and provides a 10⁷-fold increase in the number of copies of the RNA template within 1.5–2 hours [5, 43].

2.6. Rolling Circle Amplification

Rolling circle amplification (RCA) takes place on a circular (covalently closed) template with the participation of DNA polymerases phi29 or Bst with strand displacing activity [44]. As a first step, a linear ssDNA or RNA target annealed on a linear 70–100 nt long ssDNA molecule (padlock) so that the 3'- and 5'-ends of the target become adjacent and can be covalently connected by a ligase. To do this, the 10–15 nucleotide long sequences at the ends of the padlock are made complementary to the target sequence (Fig. 2E). The primer, complementary to the section of the circular template is extended by DNA polymerase with strand displacing activity to form a long (usually more than 10,000 nucleotides) ssDNA chains consisting of repeated sequences of the template. The presence of another primer in the reaction, complementary to a section of repeated sequences in the synthesized ultralong ssDNA, leads to an exponential increase in the amount of the product due to synthesis of new DNA strands on the synthesized ssDNAs (branched RCA, Fig. 2E) [5, 41].

3. BIOSENSING PLATFORMS BASED ON THE COUPLING OF CRISPR/CAS NUCLEASES WITH ISOTHERMAL AMPLIFICATION OF NUCLEIC ACIDS

3.1. CRISPR/Cas9

Cas9 was the first CRISPR/Cas nuclease used to develop a detection platform called NASBACC (Nucleic Acid Sequence-Based Amplification CRISPR-Cas Cleavage) for DNA diagnostics of Zika virus in the POCT format [45]. In NASBACC, CRISPR/Cas detection was paired with NASBA. In fact, the result of NASBA (the appearance or absence of RNA amplicons) was determined using a biosensing system based on the use of toehold switches [46]. Cas9 recognized a particular sequence in the NASBA intermediate product (dsDNA) and cleaved it that led to the occurrence of shortened RNA amplicons unable to interact with a toehold switch [45]. The use of Cas9 made it possible to discriminate Zika virus strains, which differed by a single nucleotide substitution. However, in general, the NASBACC platform turns out to be extremely complex, and the limit of detection (LOD) was only ≈ 3 fM [45].

The best values of LOD were obtained by combining Cas9 with such isothermal amplification methods as EXPAR and SDA [47, 48]. In both cases, the nickase activity is required for amplification to introduce a nick into a DNA strand that makes the elongation possible from the 3'-end by strand displacing DNA polymerase. A mutant form of Cas9 nuclease, nCas9, with the amino acid substitution Asp10Ala, which makes a nick only

in one strand of the target dsDNA, thereby triggering its amplification, was used as a nickase specifically recognizing the target sequence. The biosensing system with such selective SDA initiation was named CRISDA (CRISPR Cas9-triggered nicking endonuclease-mediated Strand Displacement Amplification) [48] and allowed the detection of single nucleotide polymorphism (SNP) associated with breast cancer in cell lines with LOD of 0.25 aM (3 copies of the target molecule per reaction). The coupling of the amplification initiation by nCas9 nuclease with the EXPAR method, known as CAS-EXPAR (CRISPR/Cas9 triggered isothermal EXponential Amplification Reaction) [47], also made it possible to achieve a comparable LOD of 0.82 aM (about 5 copies of the target molecule per reaction). In this case, ssDNA acted as a target, and a complementary target oligonucleotide with a PAM sequence (PAMmer, PAM-presenting oligonucleotide) was present in the reaction mixture. The ability of CAS-EXPAR to discriminate single nucleotide substitutions with high specificity was also shown, providing the basis for the detection of DNA methylation sites by CAS-EXPAR [47].

Along with the selective initiation of amplification, Cas9 nuclease was also used in conjunction with isothermal amplification in another format for the specific detection of target amplicons. Thus, in the method called FELUDA (FnCas9 Editor Linked Uniform Detection Assay) [49], amplicons obtained as a result of reverse transcription followed by PCR or RPA (RT-PCR, RT-RPA) carried biotin and 6-carboxyfluorescein molecules (6-carboxyfluorescein, FAM) at the ends. When the gRNA spacer recognized the section in the amplicon sequence, Cas9 broke amplicons by introducing nicks into them, thus separating the biotin/FAM pair that was detected by using immunochromatographic test strips (Lateral Flow Assay, LFA). Although in the case of RT-RPA, the LOD for SARS-CoV-2 virus was only ~400 viral genomes per reaction, in combination with RT-PCR, LOD was determined as ~10 copies of the genome per reaction that corresponds to a target concentration of ~1 aM [49]. Obviously, when combining Cas9 nucleases with one or another method of nucleic acid amplification, the LOD is completely determined by the effectiveness of the amplification *per se* (depending to a large extent on how successfully primers are selected), since Cas9 did not show collateral activity which could lead to an increase in the detected signal (for example, the level of fluorescence). The use of Cas9, however, significantly enhances the selectivity of detection. The strong dependence of the *cis*-activity of Cas9 nucleases on the presence of unpaired bases in the spacer-protospacer duplex made it possible to successfully discriminate DNA samples from donors and patients with sickle cell anemia, differing in single nucleotide substitution in the analyzed genome region [49].

3.2. CRISPR/Cas12a

The nucleases of CRISPR/Cas12 family have been most broadly used for the development of biosensing platforms, especially in combination with isothermal amplification methods such as LAMP and RPA. To date, a significant number of papers have been published on the combination of isothermal amplification with CRISPR/Cas12 nucleases, especially with Cas12a nuclease, including those demonstrating the high potential of this approach to achieve the sensitivity of nucleic acid detection at the level of single molecules. As a rule, CRISPR/Cas12a nucleases are used for selective recognition of target amplicons that leads to their *trans*-activation and enhancement of the detected signal such as fluorescence intensity. The increase in fluorescence intensity occurs due to cleavage of the large number of “reporters” by each activated nuclease — short DNA oligonucleotides carrying FAM and a fluorescence “quencher” at their termini (as the latter, chemical compounds known as Black Hole Quencher — BHQ1, BHQ2 or BHQ3 — are most often used) [50].

For the first time, Cas12a nuclease was used in combination with RPA for the detection of human papillomavirus (HPV) in 2018 [16]. The authors called this method as DETECTR (DNA Endonuclease Targeted CRISPR Trans Reporter). They demonstrated the possibility of carrying out both reactions — amplification (RPA) and Cas detection of target amplicons — simultaneously in one reaction tube [16]. Undoubtedly, this is an advantage of the developed platform, as it eliminates the potential possibility of contamination of a site where testing occurs with amplification products. DETECTR made it possible to discriminate different types of HPV based on their SNP, with LOD of several DNA targets per reaction (attomolar concentration) [16]. A year later, the utility of this approach was also shown for the detection of the bacterial pathogen, namely *M. tuberculosis* [51]. However, the assay was initially developed in a format of two test tubes: RPA and the subsequent Cas detection of target amplicons were carried out separately. After RPA was performed, an aliquot of the RPA sample was added to the test tube containing the preformed complex of Cas12a nuclease and gRNA in a reaction buffer with “reporters”, and the change in fluorescence over time was measured on a fluorimeter (instrumental detection). LOD was determined as 5 copies of bacterial genome in a microliter of the sample (or about 12 copies per 25 µl of RPA reaction) that corresponded to a subattomolar concentration [51]. In the same year, 2019, the possibility of instrument-free ultrasensitive detection by combining RPA and Cas12a nuclease was demonstrated [52]. The method was named Cas12aVDet (Cas12a based Visual Detection) and made it possible to determine the presence

of a plasmid carrying a section of the mycoplasma 16S ribosomal RNA gene with LOD of 10 aM in tested samples (6 copies of the plasmid in 25 μ l of the reaction sample). Illuminating the test tubes with blue light (for example, placing them on the working surface of a transilluminator with a wavelength of 400–500 nm or using any other light sources with the same characteristics) it was possible to read results visually, with a naked eye. The reaction mixture in the test tube with a positive result acquired an intense yellow-green color (a typical appearance of reaction tubes for such type of detection is shown in Figure 3).

It should be noted that the Cas12aVDet platform is based on conducting two reactions in the same test tube, although separately in time (sequentially). Namely, the aliquot of the mixture of gRNA and Cas12a is placed on the lid inside the test tube with RPA reaction. Once RPA is carried out, the aliquot is combined with the RPA sample by centrifugation of the tube [52]. Alternatively, the reactions can be carried out simultaneously by mixing reagents for RPA and Cas12a detection in one test tube. This approach was used, for example, in [53] to detect the bacterium *B. pseudomallei* (causing melioidosis in humans and animals). In combination with visual detection of the test result, LOD was 2.2 copies of a plasmid with a fragment of *B. pseudomallei* *T6ss* gene per reaction (or 2.2 copies of the plasmid in a microliter of the sample). When detecting

bacterial DNA, LODs were 12.5 and 61.5 colony forming units (CFU) in 1 ml of the bacterial culture or blood, respectively (0.013 and 0.06 CFU per reaction) [53]. The comparable LODs for *E. coli* pathogenic strain O157:H7 — about 1.2 CFU in 1 ml of bacterial culture and 4.8 CFU in 1 g of plant material (0.006 and 0.024 CFU per reaction) — were also determined when combining Cas12a detection with another method of isothermal amplification, LAMP [54]. In this case, the reactions were carried out sequentially in different test tubes due to the incompatibility of their temperature regimes.

In addition to the “reporters” carrying FAM and BHQ1 at their termini, other fluorophore-“quencher” combinations can be employed. In [55], DNA oligonucleotides carrying ROX (carboxy-X-rhodamine) and BHQ2 at the termini were used as “reporters”. For this “reporter”, the color of the reaction mixture changed from blue to red upon a positive test that could be determined visually without using any special lighting. The test was performed in a single test tube (RPA and Cas12a-detection of target amplicons as in [52]) and allowed detection of bacterial pathogen *P. multocida* (causes pasteurellosis in humans and animals) in clinical samples with sensitivity not inferior to PCR. Using a plasmid carrying a fragment of the *Kmt1* gene of *P. multocida*, LOD was 2 copies of plasmid in 1 μ l of sample (4 copies in a test tube) [55].

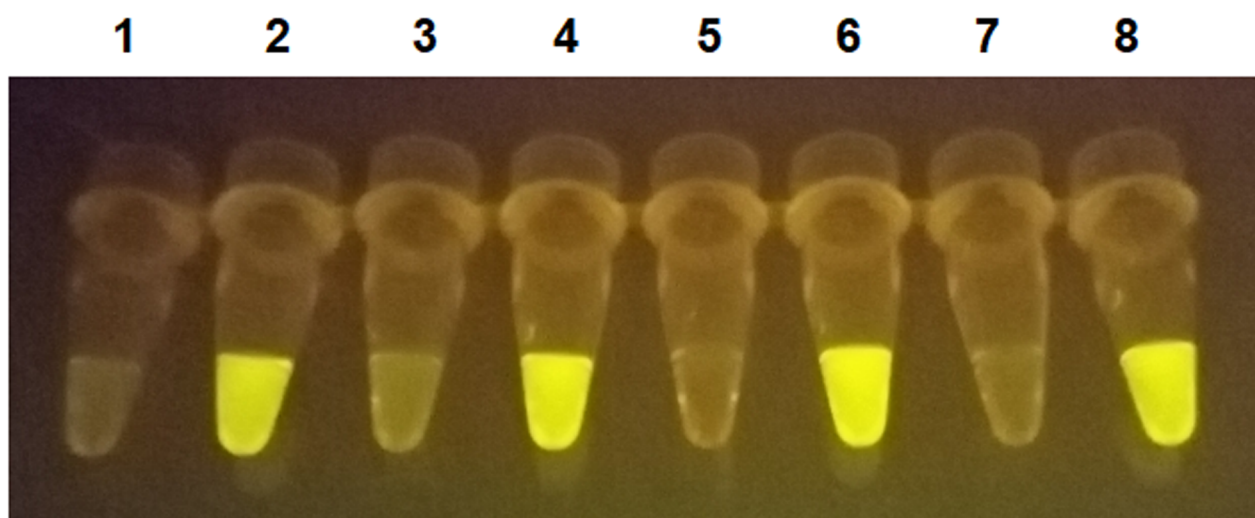


Figure 3. An example of a visual (non-instrumental) detection of a test result. A section of the genomic DNA of phytopathogen *Dickeya solani* was amplified by RPA method and the presence of target amplicons was determined using Cas12a nuclease and DNA oligonucleotides carrying FAM and BHQ1. The test tubes are illuminated with blue light. Odd numbers are control (RPA in the absence of *D. solani* genomic DNA), even numbers are RPA in the presence of 10 copies of the *D. solani* genome in the reaction. The results of four independent tests (unpublished results of the authors) are shown.

Alongside with the colorimetric method based on the color change of the reaction mixture during test tube illumination with blue light, it was shown that LFA could also be used as an instrument-free detection method when combining Cas12a nuclease with isothermal amplification. Thus, the combination of LAMP with Cas12a detection of target amplicons (LAMP amplicons) made it possible to identify the bacterial pathogen of nosocomial infections *P. aeruginosa* with ultra-high sensitivity [56]. The method was named CIA (CRISPR/Cas and loop-mediated Isothermal Amplification) and for a plasmid carrying a specific section of the acetyltransferase gene of this bacterium, it had LOD of 1 aM in the tested samples (or 1 copy of plasmid in the LAMP reaction). For bacterial DNA of *P. aeruginosa*, LOD was 3.4 aM [56]. It should be noted that for visualization, the authors used immunochromatographic test strips of their own manufacture, where the test zone represented poly(dA) oligonucleotides immobilized on a porous membrane, and the control zone represented biotinylated antibodies. In this case, gold nanoparticles carried streptavidin molecules on the surface, while biotinylated DNA oligonucleotides with a site of preferred cleavage by *trans*-activated Cas12a nuclease (TTATT) at the 3'-end and a poly(dT) site at the 5'-end were used as "reporters" [56]. Along with this, commercial test strips of various types can be used for non-instrumental detection. For example, in [57], the authors used commercial LFA designed for pregnancy testing. Human chorionic gonadotropin (HCG) was conjugated with large dsDNA molecules through an ssDNA linker, forming a covalent complex that, due to its size, could not pass through the pores of the test strip membrane. *Trans*-activation of Cas12a nuclease, caused by the occurrence of target amplicons, led to the release of HCG, which was determined using the test strip. This detection method was called CLIPON (CRISPR and Large DNA assembly Induced Pregnancy strips for signal-ON detection). In combination with RT-RPA, the method allowed to detect SARS-CoV-2 virus genome with LOD of 1 copy/μl [57]. For non-instrumental detection, the commercial test strips specifically designed to detect DNA fragments carrying both FAM and biotin molecules (usually, they are amplicons generated by using FAM and biotin-labeled primers, as, for example, in [58]) were also employed. Using such test strips, LOD for determining SARS-CoV-2 virus in artificial and clinical samples by a combination of RT-RPA with Cas12a detection of target amplicons was 2.5 copies/μl (or about 10 copies of SARS-CoV-2 genome in the reaction mixture) [59]. In this case, all reactions were carried out in the format of a single test tube, similar to that in [52].

The use of Cas12a nucleases for the selective detection of target amplicons requires the presence of PAM that may impose additional restrictions

on the selection of primers for DNA amplification. As shown in [60], this limitation can be circumvented by using Cas12a mutants with relaxed PAM requirements. Thus, the recombinant nuclease Mb2Cas12a-RRVRR obtained by directed mutagenesis, which is a mutant form (substitutions D156R, G532R, K538V, Y542R, and K595R) of Cas12a nuclease found in one of *M. bovoculi* strains, requires the presence of NTTV, NTCV and NCTV sequences as PAMs in amplicons. Its use in coupling Cas12a detection with RPA made it possible to detect a target DNA with a detection limit of 2.4 copies in the reaction mixture. RPA and Cas12a detection was performed sequentially, but in one test tube, and the test result was determined using commercial test strips. Interestingly, this LOD has been reached at 35°C, which allows — as shown by the authors [60] to conduct testing without using a thermoblock, simply by placing a test tube in axilla. It should be noted that, when RPA and Cas12a detection are performed simultaneously in the same test tube, high nuclease *trans*-activity can have a negative effect, namely, can inhibit amplification (presumably due to the cleavage of primers and single-stranded sites in DNA target, formed when primers are incorporated by recombinase into dsDNA), thus reducing the overall sensitivity of the method [61, 62]. It is likely that the *cis*-activity of Cas12a nuclease, acquired in the presence of PAM, can also affect the effectiveness of RPA due to the cleavage of the target dsDNA and dsDNA amplicons. In this case, the use of suboptimal PAM sequences makes it possible to reduce the activity of Cas12a nuclease and, in general, increase the sensitivity of testing due to more effective amplification of the target. As shown in [63], Cas12a nuclease from *Lachnospiraceae* bacterium ND2006 can use the sequences TCC, TCG, CCT, CTC, GTC, and CTG as suboptimal PAMs. Their presence in amplicons instead of canonical PAM (TTTN) made it possible to optimize detection, reaching LOD of 0.2 and 0.4 copies/μl for DNA and RNA targets (in the latter case, RT-RPA was used) that corresponded to 1 and 2 copies of the target in the reaction mixture [63]. The amplification and Cas12a detection were carried out simultaneously in one test tube, and the test result was determined visually when the test tube was illuminated with blue light.

Simultaneous detection of multiple targets (multiplex detection) is an undoubted advantage of a biosensing platform. The feasibility of such detection by combining multiplex RPA and Cas12a nuclease was shown in [64]. The products of simultaneous amplification of fragments of the *TcdA* and *TcdB* genes of bacterium *C. difficile* (encode *C. difficile* toxins A and B, respectively), the causative agent of pseudomembranous colitis, were detected in different test tubes, each of which contained a Cas12a nuclease complex with gRNA recognizing the target sequence either in *TcdA* gene or in *TcdB* gene. Visualization of the result of Cas12a *trans*-activation

was determined both by the instrumental method (on a fluorimeter) and by non-instrumental methods — visually by either the color change of the test tube upon its illumination with blue light or using commercial test strips. In all cases, LOD was 10 and 1 copies in the reaction (RPA) mixture for *TcdA* and *TcdB* gene, respectively, when using a plasmid with gene fragments. In testing clinical samples, the sensitivity of the method was not inferior to that in PCR [64].

In addition to RPA and LAMP, the ultrahigh detection sensitivity was demonstrated by combining Cas12a and RCA — LODs for microRNA (miR-21) and parvovirus B19 (causing the development of chronic anemia in humans) detection were 0.83 and 0.52 aM of the target in the sample, respectively [65]. However, the overall detection scheme was rather complex. Amplification was performed in a separate tube and an aliquot containing the RCA product was added to the test tube with Cas12a nuclease. In the reaction mixture with Cas12a nuclease, a DNA oligonucleotide was present, which was complementary to a portion of the repeating sequence in ultralong ssDNA — the RCA product. As a result, the numerous duplexes were formed, thus representing DNA sites recognized by the gRNA spacer that led to the acquirement of *trans*-activity by Cas12a nuclease. A second DNA oligonucleotide (universal blocker probe, BP) was also present in the reaction mixture and was cleaved by the activated Cas12a. The aliquot containing the reaction products was mixed with an equal volume of a solution containing “reporters” (universal reporter probe, RP) — a DNA oligonucleotide complementary to BP and carrying at its terminus a dye, methylene blue, with high electrochemical activity. The resulting mixture was placed on the surface of a carbon electrode modified with reduced graphene oxide. If BP was degraded, then the BP/RP duplex was not formed, RP was adsorbed on the electrode surface and an electrochemical oxidation signal of methylene blue was detected. Otherwise, a duplex was formed, which had no affinity for the electrode surface and, accordingly, there was no oxidation signal [65].

Thus, to date, numerous examples have shown the feasibility of developing diagnostic biosensing platforms by combining methods of nucleic acid isothermal amplification, such as RPA and LAMP, with detection of target amplification products with Cas12a nuclease. It should be noted that in all the above-mentioned works, exceptional detection selectivity was also demonstrated. With high sensitivity (at the level of single molecules), such platforms will be easy to use. This is important for DNA diagnostics in the PONT format: the testing can be carried out in a single reaction tube without using sophisticated equipment, and the result can be determined either visually by the color change of the reaction mixture, or by using commercial test strips.

3.3. CRISPR/Cas12b

Cas12b nuclease obtained from thermophilic bacteria can exhibit nuclease activity at higher temperatures than Cas12a. This property of Cas12b nuclease proved to be in demand for the development of diagnostic biosensing platforms based on combining Cas detection with LAMP, since it potentially allowed testing in the format of a single test tube. The use of Cas12b nuclease (obtained from the thermophilic bacterium *A. acidoterrestris*) was first reported in 2019 [66]. However, this particular nuclease had acceptable *trans*-activity only at temperatures not exceeding 55°C that significantly limited the effectiveness of LAMP and led to a strong increase of testing time. Later, Cas12b from a bacterium of the *Brevibacillus* family was employed that allowed LAMP to be carried out at a higher temperature, 60–62°C (the method was called HOLMESv2) [67]. Recently, a recombinant Cas12b nuclease modified by genetic engineering has been proposed, which has acceptable *trans*-activity up to a temperature of 67°C [68], thus allowing LAMP to be carried out in the temperature range optimal for the method, 60–65°C.

As in the case of Cas12a, the use of Cas12b nuclease makes it possible to achieve ultra-high detection sensitivity. Thus, in [69], using Cas12b from the thermophilic bacterium *A. acidiphilus*, LOD of 10 copies/μl (10 copies in the reaction mixture) was obtained for the detection of human hepatitis B virus, allowing to determine 25 copies of the virus genome in 1 ml of blood. The testing was carried out in one test tube (LAMP and Cas detection took place simultaneously) at 60°C, followed by instrumental (using a fluorimeter) measurement of fluorescence intensity as the reaction output [69]. The high sensitivity of tests based on a combination of LAMP and Cas12b nuclease has also been demonstrated in other studies, for example [70–72]. In [70] it was shown that the detection of parvovirus (causing enteritis in dogs) could be carried out both in the format of two and one test tube. In both cases, the overall detection sensitivity was characterized by LOD of about 1 copy of target DNA in reaction mixture (0.1 copy/μl) that was 100 times less than that in the case of PCR or LAMP alone. The LOD for monkey pox virus (causing zoonotic infection which can be transmitted to humans from animals or through close contact between humans) was found to be 10 copies of virus genomic DNA in a reaction mixture, using the LAMP/Cas12b system, determining the test result with a test strip [71]. The same LOD was obtained for detecting bacterial pathogen, *P. aeruginosa*, by a combination of LAMP and Cas12b in the format of a single test tube [72].

Thus, the use of Cas12b nuclease makes it possible to combine Cas12b detection with LAMP, providing testing in a single-tube format with sensitivity at the level of the attomolar concentration of the target.

Interestingly, Cas12b was also used in combination with RPA: the limit for determining synthetic DNA fragments mimicking a section of the genome of highly oncogenic type of HPV, the HPV16, was 1 aM [73].

3.4. CRISPR/Cas14 (Cas12f)

Despite the fact that Cas14 nuclease has a small size in comparison with other Cas nucleases, which is considered as an advantage [74], the nuclease has not yet been widely used for the development of diagnostic biosensing platforms based on CRISPR/Cas nucleases. This may be due to the fact that, in practical terms, the larger size of Cas12a and Cas12b nucleases did not prove to be a critical factor for their successful use in diagnostic biosensing systems. Nevertheless, the use of Cas14 nuclease for the selective detection of RPA amplicons has shown that it is possible to achieve low values of LOD [75]. Using plasmids containing fragments of either genes of *M. pneumoniae* or hepatitis B virus as a target, the LOD values were 10 copies of plasmid in 1 μ l (20 copies in the RPA sample). The RPA and Cas14 detection were carried out sequentially in different test tubes, and the result was determined by measuring the level of fluorescence on a fluorimeter [75].

3.5. CRISPR/Cas3

Like Cas14, Cas3 nuclease is currently not widely employed to develop diagnostic biosensing systems that is most likely due to the need of complex riboprotein complexes requiring expression and purification. Indeed, in [76], to design a diagnostic platform based on Cas3 nuclease (called CONAN — Cas3-Operated Nucleic Acid detection), the authors used a multicomponent riboprotein complex EcoCascade (including crRNA and several Cas proteins from *E. coli*) obtained by chromatographic purification after co-expression in the baculovirus vector system, and recombinant EcoCas3 nuclease from *E. coli*. In combination with RPA (with sequential reactions in two test tubes), it was possible to achieve the high sensitivity of detection for synthetic DNA fragments representing sections of human EMX1 gene and mouse *Tyr* gene: LODs were at the level of single copies of fragments (attomolar concentration). However, for synthetic fragments representing cDNA regions of the genomes of influenza virus H1N1 and SARS-CoV-2 virus, it was not possible to achieve high sensitivity that the authors attributed to an unsuccessful choice of primers for RPA (known sequences of primers used for PCR detection of these viruses were employed). CONAN made it possible to perform detection either using a fluorimeter or in an instrument-free format by using test strips [76]. In general, it seems that the coupling of Cas3 nuclease with isothermal

amplification does not provide any significant advantages compared to Cas12 nucleases, while the need to obtain and purify multicomponent riboprotein complexes significantly reduces the potential of its practical application.

3.6. CRISPR/Cas13a

Cas13a was the first CRISPR/Cas nuclease used in 2017 to develop a diagnostic biosensing platform named SHERLOCK (Specific High-sensitivity Enzymatic Reporter unLOCKing) [15]. SHERLOCK is based on conducting RT-RPA with primers, the sequence of one of which is supplemented by a T7 RNA polymerase promoter, subsequent synthesis of RNA fragments on RPA amplicons as a template, and selective detection of target RNA fragments by the gRNA/Cas13a complex. Short RNA oligonucleotides carrying FAM and a “quencher” at their termini were used as “reporters”. The platform was further developed as SHERLOCKv2 [77] and HUDSON (Heating Unexploded Diagnostic Samples to Obliterate Nucleases) [18] with the capabilities of testing in a single test tube with lyophilized reagents and instrument-free detection of the reaction result by using commercial test strips (in this case, the “reporters” carried FAM and biotin at the termini), as well as with the possibility of multiplex testing (SHERLOCKv2). The LODs for the determination of dengue and Zika viruses at the level of attomolar concentrations (1–2 copies of the virus per reaction) were achieved [18, 77].

The detection schemes of the SHERLOCK, SHERLOCKv2, and HUDSON diagnostic platforms became widely used to develop highly sensitive and selective tests for the detection of various pathogens. Sequentially performing RT-RPA, RNA synthesis with T7 RNA polymerase and Cas13a detection of target RNA fragments with visual registration of the reaction result (the test tube was illuminated with blue light), the LOD of 10 copies per reaction was reached for transmissible gastroenteritis virus [78]. The test showed high selectivity and was not inferior in sensitivity to the PCR test. Using sequentially performed reactions, the bacterial pathogen *V. parahaemolyticus* was detected with LOD of 1 copy/ μ l (1 copy of genome per RPA reaction) using commercial test strips to visualize the test result [79]. It was also shown that testing could be performed in a single test tube: the H9N2 avian influenza virus was detected with LOD of 1 copy/ μ l (5 copies of virus genome in reaction sample) with high selectivity by measuring the fluorescence level in the test tube on a fluorimeter [80].

Along with RPA, attempts have been made to couple Cas13a detection with NASBA. Such coupling has an undoubted advantage in that the NASBA product is RNA amplicons, which can be recognized by the gRNA/Cas13a complex, so that there is no need for synthesis of RNA fragments. Another advantage

is that transcripts can be detected directly, the number of which can exceed the number of copies of the gene by orders of magnitude, while eliminating the need for a reverse transcription step to obtain cDNA. However, to date, diagnostic biosensing platforms based on a combination of NASBA and Cas13a detection have not shown sensitivity comparable to that of the RPA/Cas13a platform. For example, when combining NASBA and Cas13a detection in the format of a single test tube, the LODs for RNA fragments representing different regions of the SARS-CoV-2 virus genome were 20–200 aM [81]. In another study, the limit for determining the number of SARS-CoV-2 virus genomes was 40 aM when combining NASBA and Cas13a (the method was called CASCADE — CRISPR/CAS-based Colorimetric nucleic Acid DETection) [82]. In CASCADE, the test result was determined visually, for which nanoparticles functionalized with DNA oligonucleotides were used. Degradation of DNA oligonucleotides immobilized on nanoparticles as a result of the acquisition of *trans*-activity by Cas13a nuclease led to aggregation of nanoparticles, causing a change in the color of the reaction mixture from red to purple. Interestingly, the use of this method of colorimetric detection of the test result when coupling Cas13a with RT-RPA on the same object gave LOD of only 3 fM [82]. In another work, a complex signal amplification scheme based on the synthesis of the so-called “broccoli” aptamer, initiated by activated Cas13a, was used [83]. The “broccoli” aptamer is an RNA aptamer that is capable of forming stable complexes with the fluorescent dye DFHBI (3,5-difluoro-4-hydroxy-benzylidene imidazolinone), causing its fluorescent glow [84]. After completing NASBA (the target was a section of SARS-CoV-2 virus genome), amplification products were added to a test sample with an gRNA/Cas13a complex. The test sample also contained a DNA oligonucleotide with an insert representing two uridines. The *trans*-activated Cas13a cleaved the oligonucleotide between uridines, turning it into a primer, the elongation of which on another DNA oligonucleotide (also present in the test sample) created a template for the synthesis of “broccoli” aptamer by T7 RNA polymerase. The occurrence of the “broccoli” aptamer in the system leads to the binding of DFHBI and the development of fluorescence which was recorded on a fluorimeter [83]. Despite the use of such complex approach involving several successive stages of signal amplification LOD was only 216 aM [83].

CONCLUSIONS

The design of biosensing platforms based on CRISPR/Cas nucleases appears to be one of the most promising directions for developing non-laboratory DNA diagnostics, capable of providing detection sensitivity at the level of single molecules (attomolar and subattomolar concentrations of a target

in a sample). The use of CRISPR/Cas nucleases ensures the specificity of detection that is determined by the selective recognition of target amplicons by the gRNA/Cas nuclease complex. CRISPR/Cas nucleases Cas12a, Cas12b, and Cas13a have become most widespread in the development of diagnostic biosensing platforms that is associated with the possibility of enhancing the detected signal by tens or hundred times when they acquire *trans*-activity. These nucleases have shown compatibility with widely used methods of isothermal amplification of nucleic acids (LAMP, RPA, and NASBA) and non-instrumental detection methods (visual by changing the color of the test sample and using commercial test strips) that meets the requirements of PONT. Testing is possible in the format of a single test tube that eliminates the problem of contamination of samples with amplification products. Despite its smaller molecular size, Cas14 nuclease (Cas12f) has not received any significant use in the development of biosensing platforms. The inability to amplify the signal in the case of Cas9 nucleases is thought to be the main limiting factor for their use in DNA diagnostics, despite their commercial availability due to the widespread use in genomic editing. However, the very recently discovered ability of Cas9 nucleases to acquire *trans*-activity under certain conditions *in vitro* may change their place in the development of biosensing platforms. The use of Cas3 nuclease does not seem promising due to the complexity of the enzymatic system without obvious advantages in detection sensitivity. It seems that the further development of this area will be primarily associated with the creation and distribution of genetically modified Cas12 nucleases with reduced requirements for the presence of PAM or their complete absence, as well as with increased thermal stability. The development of new approaches to enhance the signal generated by *trans*-activated Cas nuclease will also be the focus of research.

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COMPLIANCE WITH ETHICAL STANDARDS

This article does not contain any research involving humans or the use of animals as objects.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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**БИОСЕНСОРНЫЕ ПЛАТФОРМЫ ДЛЯ ДНК-ДИАГНОСТИКИ
НА ОСНОВЕ CRISPR/Cas-НУКЛЕАЗ: НА ПУТИ К ДЕТЕКЦИИ НУКЛЕИНОВЫХ КИСЛОТ
НА УРОВНЕ ЕДИНИЧНЫХ МОЛЕКУЛ ВО ВНЕЛАБОРАТОРНЫХ УСЛОВИЯХ**

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В последние годы наметился бурный рост исследований с использованием CRISPR/Cas-нуклеаз для разработки систем ДНК-диагностики во внелабораторных условиях (*англ.* point-of-need testing, PONT), началом развития которых послужила разработка в 2017–2018 гг. первых диагностических платформ, известных как DETECTR и SHERLOCK. В их основе лежит сочетание методов изотермической амплификации нуклеиновых кислот с селективной CRISPR/Cas-детекцией целевых ампликонов. Это позволяет значительно улучшить чувствительность и специфичность PONT, сопоставимых с (или даже превосходящими) чувствительностью и специфичностью полимеразной цепной реакции, являющейся “золотым стандартом” ДНК-диагностики. В обзоре рассмотрены современные подходы к сопряжению CRISPR/Cas-детекции с использованием нуклеаз Cas9, Cas12a, Cas12b, Cas13a, Cas14 и Cas3 с различными методами изотермической амплификации нуклеиновых кислот, с акцентом на работы, в которых достигнута чувствительность на уровне единичных молекул (субатомолярных концентраций мишени). Коротко рассмотрены свойства CRISPR/Cas-нуклеаз, используемых для целей ДНК-диагностики, и особенности методов изотермической амплификации нуклеиновых кислот, применяемых сегодня при разработке диагностических биосенсорных платформ. Обсуждаются наиболее перспективные направления развития ДНК-диагностики с использованием CRISPR/Cas-нуклеаз.

Полный текст статьи на русском языке доступен на сайте журнала (<http://pbmc.ibmc.msk.ru>).

Ключевые слова: CRISPR/Cas-нуклеазы; детекция нуклеиновых кислот; единичные молекулы; ДНК-диагностика

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