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MINIREVIEW

Recent Advances in CRISPR-Cas Technologies for Synthetic Biology

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Abstract
With developments in synthetic biology, "engineering biology" has emerged through standardization and platformization based on hierarchical, orthogonal, and modularized biological systems. Genome engineering is necessary to manufacture and design synthetic cells with desired functions by using bioparts obtained from sequence databases. Among various tools, the CRISPR-Cas system is modularly composed of guide RNA and Cas nuclease; therefore, it is convenient for editing the genome freely. Recently, various strategies have been developed to accurately edit the genome at a single nucleotide level. Furthermore, CRISPR-Cas technology has been extended to molecular diagnostics for nucleic acids and detection of pathogens, including disease-causing viruses. Moreover, CRISPR technology, which can precisely control the expression of specific genes in cells, is evolving to find the target of metabolic biotechnology. In this review, we summarize the status of various CRISPR technologies that can be applied to synthetic biology and discuss the development of synthetic biology combined with CRISPR technology in microbiology.

Keywords CRISPR-Cas technologies · Synthetic biology · Microbiology

Introduction

Advances in biotechnology are essential for sustainable human life to solve various problems caused by population growth in the fields of environment, food, energy, and health care. Synthetic biology has developed remarkably to maximize the utilization of biological systems through standardization and platformization (Purnick & Weiss, 2009). Life systems replicate and grow as programmed in the genome; they can also provide the intended values. Therefore, a new genome should be efficiently constructed for a desired purpose. With the development of sequencing technology, the nucleotide sequences that make up and program living organisms have been revealed tremendously, and new systems can be designed by extracting necessary parts, circuits, and pathways from the database (Chen et al., 2012; Quince et al., 2017).

Since the genetic code is universal from microorganisms to higher organisms, genes obtained from living organisms can be recombined in new cells (Ostrov et al., 2020). Based on the rapid in vitro chemical synthesis of oligonucleotides, several DNA assembly methods have been developed (Hughes & Ellington, 2017). Among them, sequence homology-based methods such as Gateway, circular polymerase extension cloning (CPEC), and Gibson assembly have been applied to develop a larger DNA assembly (Chao et al., 2015; Gibson et al., 2010; Wang et al., 2018a). A megabase-sized genome can be synthesized in cells, and a synthetic cell can be built for microbial chassis cells (Hutchison et al., 2016).

Various functions, including environmental signal sensing, intracellular signal transduction, and biochemical production, can be implemented in synthetic cells (Brophy & Voigt, 2014). Synthetic genetic circuits, such as a toggle switch (Gardner et al., 2000), an oscillator (Stricker et al., 2008), feedback loops (Dahl et al., 2013), and a Boolean logic gate (Green et al., 2017), can control cell performance and behavior. Moreover, microorganisms are reprogrammed to diagnose and treat diseases (Riglar & Silver, 2018) and can be utilized as biosensors capable of detecting metabolites, enzyme products, and harmful substances (Kim et al., 2016a). In addition, reprogrammed microorganisms are used to produce desired substances from synthetic cells in the fields of bioenergy, chemistry, and medicine (Nielsen & Keasling, 2016). For example, the biosynthetic

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pathway from *Artemisia annua* L. (sweet wormwood) can be transplanted to *Saccharomyces cerevisiae* to produce artemisinic acid, an antimalarial drug precursor in microbial cell factories (Ro et al., 2006).

Synthetic biology tools have been developed to design and optimize living systems (Leon-Buitimea et al., 2022). Among them, CRISPR-Cas, an adaptive immune system of microorganisms, has been developed as a genome editing technology (Jinek et al., 2012). CRISPR gene scissors are divided into two modules: nucleolytic protein and target recognition RNA; therefore, they can be used and applied more synthetic-biologically than any other tool (Clarke et al., 2021). Unlike restriction enzymes, any target sequence can be easily and freely designed, and CRISPR-Cas can operate from bacteria to higher organisms.

In microbiology, CRISPR technology has advanced in the following directions. CRISPR genome editing tools have been developed in various microorganisms (Lee & Lee, 2021). The function of recognizing specific sequences in CRISPR-Cas has been expanded and applied to molecular diagnosis (Kim et al., 2021). In addition, with nucleolytic activity-free Cas proteins, gene expression and biosynthetic circuits in synthetic cells can be precisely regulated (Santos-Moreno & Schaerli, 2020). Here, we review the latest trends in CRISPR technology, an essential tool for the development of synthetic biology, and summarize how it is applied to microbiology. We also discuss the prospects for the development of CRISPR-Cas technologies in microbiology.

CRISPR-Mediated Genome Editing

The CRISPR-Cas system, which exhibits adaptive immunity in microorganisms, is modularized with single guide RNA (sgRNA) that recognizes nucleic acid targets and Cas protein that causes cleavage (Mali et al., 2013). The target nucleotide sequence can be freely modified on the basis of the change in the RNA sequence; it is also used as a genome editing tool in many organisms, including bacteria and yeasts. Studies on genome editing with CRISPR-Cas in various microorganisms, such as archaea, bacteria, and yeasts, are summarized in Table 1.

CRISPR-Cas9-mediated gene editing was first reported in *Escherichia coli* among microorganisms (Jiang et al., 2013). In *Staphylococcus aureus*, gene deletion, insertion, and base substitution are performed using a single plasmid containing Cas9, sgRNA, λ-Red recombinase, and donor DNA (Chen et al., 2017). The CRISPR-Cas9 system is also used in *Trichoderma reesei*, a filamentous fungus, and a relatively high homologous recombination efficiency (> 93%) is achieved when the length of the donor DNA homology arm is 200 bp (Liu et al., 2015). Genes are edited with > 70% efficiency in *Streptomyces* by using a single plasmid containing Cas9, sgRNAs, and donor templates (Cobb et al., 2015). Up to three heterologous genes are simultaneously inserted into various regions of the *E. coli* genome by using CRISPR-Cpf1 and λ-Red recombinase (Ao et al., 2018).

Recently, Cas12f1, which has a relatively smaller gene than Cas9 and Cpf1, was discovered from metagenomic data (Harrington et al., 2018); studies have reported gene deletion in *E. coli* and *Bacillus anthracis* by using CRISPR-Cas12f1 (Okano et al., 2021; Wang et al., 2022c). In addition, new class of RNA-guided nucleases such as IscB and TnpB were discovered by exploring the evolutionary origin of Cas9 and Cpf1 nucleases (Karvelis et al., 2021; Schuler et al., 2022). IscB and TnpB, which are considered ancestors of Cas9 and Cas12 nucleases, have lower editing efficiency. However, since the protein size is small, it has the advantage of being used for gene therapy.

Target Sequence Identification

As CRISPR-mediated genome editing was studied, an off-target effect of cutting similar sequences outside the target was observed in the eukaryotic system with high genome complexity. In some cases, cleavage occurs at undesired locations in the genome, and this process has been recognized as an obstacle to editing (Lin et al., 2014). In order to solve this problem in eukaryotic cells, studies have been conducted to increase target specificity and editing efficiency by engineering guide RNA or Cas nuclease.

The chemical modification of the crRNA terminus of Cpf1, including methylation and fluorination, improves crRNA stability and editing efficiency (McMahon et al., 2018). 2′-*O*-methyl-3′-phosphonoacetate modification of ribose in crRNA improves Cas9 function and target specificity in some cases (Ryan et al., 2018). The extension of the 5′ end of crRNA in Cpf1 enhances the efficiency of genome editing such as gene knockout and homology-directed repair (Park et al., 2018a), and uridylation of the 3′ end improves the efficiency of indel editing by using Cpf1 (Moon et al., 2018). When a part of the spacer in crRNA is replaced with DNA, the editing efficiency is improved and the off-target effect is reduced by changing the binding energy to the target (Kim et al., 2020b).

Cas protein engineering has been reported as another strategy to improve target specificity. For example, on-target activity can be improved by fusing a chromatin-modulating peptide with Cas9 (Ding et al., 2019). In another study, FokI nuclease is fused with deactivated Cas9 (dCas9) to form nicks at different positions and strands, thereby reducing the off-target effect (Ding et al., 2019; Guilinger et al., 2014).

Cas9 has a PAM strand-specific RuvC nuclease domain and a target strand-specific HNH nuclease domain (Jinek et al., 2012). When Asp10 or His840 (the catalytic residues of RuvC and HNH domains, respectively) is substituted with

Table 1 CRISPR-mediated genome editing in microorganisms

Organism	Cas protein	Species	Donor DNA	Types of edits (bp)	Feature	References
Archaea	Cas9	*Methanosarcina acetivorans*	Plasmid None*	D (34–2624) I (2526 and 3045)	Multiple two different gene deletion using single plasmid harboring four sgRNAs	Nayak and Metcalf (2017)
		Sulfolobus islandicus	Plasmid	D (442) I (18) S (9)	X-Gal blue-white colony screening	Li et al. (2016b)
Bacteria	Cas9	*Bacillus subtilis*	Plasmid	D (4100–25,000) S (3)	PAM substitution	Altenbuchner (2016)
		Clostridium acetobutylicum	Plasmid	D (306) I (2985) S (2)	Two-plasmid system (Cas9 plasmid, sgRNA + donor DNA plasmid)	Wasels et al. (2017)
		Escherichia coli	Oligo	S (1)	Target-mismatched sgRNA	Lee et al. (2020a)
		E. coli	Oligo	D (1) I (1) S (1)	5′-truncated sgRNA	Lee et al. (2021)
	nCas9	*B. licheniformis*	Plasmid	D (997–40,909) I (1335)	Simultaneous multiple-gene disruption	Li et al. (2018a)
		B. subtilis	Plasmid	D (1000–20,500) I (1000–2000) S (1)	Improving editing efficiency by using a *ligD*-deficient strain	Liu et al. (2019a)
		C. beijerinckii	Plasmid	D (20–1149)	All-in-one plasmid (nCas9, sgRNA, and donor DNA)	Li et al. (2016a)
	Cas12a	*Corynebacterium glutamicum*	Oligo	S (1)	Target-mismatched crRNA	Kim et al. (2020c)
		E. coli	Oligo	D (1) I (1) S (1)	3′-truncated crRNA	Lee et al. (2022b)
		Mycobacterium smegmatis	Oligo	D (1) I (1) S (2)	gp60, gp61-mediated ssDNA recombineering, X-Gal blue-white colony screening	Yan et al. (2017)
			PCR product	D (2–4000) I (642–1000)		
	Cas12f1	*B. anthracis*	Plasmid	D (1242–14,600)	Co-expressing AsCas12f1 and I-*Sce*I nuclease	Wang et al. (2022c)
		E. coli	Plasmid	D (891–2145)		Okano et al. (2021)
Fungi	Cas9	*Aspergillus fumigatus*	None	D (1) I (219)	Verification of editing efficiency with albino phenotype	Fuller et al. (2015)
		Penicillium chrysogenum	PCR product	D (1275–23,439) I (1449–3170)	Genome editing using preassembled sgRNA/Cas9 ribonucleoprotein	Pohl et al. (2016)
		Trichoderma reesei	PCR product	D (2,244)	Multiple gene deletion using separate gRNA and donor DNA plasmids	Liu et al. (2015)
	Cas12a	*A. aculeatus*	None	D (7) I (2)	crRNA-flanking tRNA fusion transcript with U3 Pol III promoter	Abdulrachman et al. (2021)
		A. nidulans	Oligo PCR product	I (6) I (678)	Marker-free genome editing with NHEJ-deficient strain	Vanegas et al. (2019)

Table 1 (continued)

Organism	Cas protein	Species	Donor DNA	Types of edits (bp)	Feature	References
Yeast	Cas9	*Kluyveromyces lactis*	PCR	I (3574)	Multiplexed gene integration by single plasmid harboring multiple sgRNA	Horwitz et al. (2015)
		Pichia pastoris	PCR	D (100 ~ 1800)	ribozyme strategy for gRNA expression with self-splicing RNA element	Weninger et al. (2016)
		Scheffersomyces stipitis	PCR	D (1,463)	Elimination of the NHEJ mechanism by ku70/ku80 genes to improve homologous recombination	Cao et al. (2018)
		Yarrowia lipolytica	PCR	I (1026)	Expression of sgRNA with tRNA-based synthetic promoters	Schwartz et al. (2016)
	Cas12a	*Saccharomyces cerevisiae*	PCR	I (8429)	Evaluation of editing efficiency using three Cpf1 orthologues	Verwaal et al. (2018)
		Schizosaccharomyces pombe	PCR	D (1659)	Multiple crRNA array using a strong constitutive pol II promoter	Zhao and Boeke (2020)
		Y. lipolytica	None	D (2)	Improving editing efficiency by addition of poly-thymidine to the 3′-end of crRNA	Yang et al. (2020)

*NHEJ-mediated genome editing

Ala, Cas9 nickase (nCas9), which cleaves a single strand of target DNA, can be generated (Cong et al., 2013; Gasiunas et al., 2012). The CRISPR-nCas9 (D10A) system is used to insert or delete gene cassettes of 1 kb or less with an editing efficiency of up to 100% in *Lactobacillus casei* (Song et al., 2017). Since nCas9 was designed to target two adjacent sites on different strands, it has been used as a genome editing method with improved target specificity by targeting more nucleotide sequences and resulting in double-strand break (DSB) (Cho et al., 2014; Ran et al., 2013). Multiple nicks are formed at different positions and strands in *E. coli* to perform a final 133 kb deletion (Standage-Beier et al., 2015).

In mammalian cells, the development of technologies for detecting off-target effects remains a key challenge. Recently, strategies for detecting off-target effects have been developed. Various methods for detecting off-target effects including Web-based prediction tools, CHIP-seq, GUIDE-seq, and HTGTS have been developed and applied (Zhang et al., 2015). Recently, unwanted mutations can be avoided by profiling the off-target effect of nucleases including Cas9 through Digenome-seq (Kim et al., 2015).

The protospacer adjacent motif (PAM) sequence is located near the target DNA and helps the bacterial adaptive immune CRISPR-Cas system to discriminate between self- and non-self-target sequences (Marraffini & Sontheimer, 2010). However, PAM sequences limit the range of target sequences that Cas proteins can recognize in genome editing. Therefore, studies have been performed to relieve the restriction of the PAM sequence by engineering Cas nucleases and solve this problem. For example, a Cas9 variant (xCas9) that can recognize PAMs of different sequences, such as NG, GAA, and GAT, has been developed through phage-assisted continuous evolution [PACE; (Hu et al., 2018)]. Cas9-NG that recognizes 5′-NG as a PAM sequence has also been designed by eliminating the dependence of the Cas9 protein on the third guanine of 5′-NGG, thereby expanding the range of target selection (Nishimasu et al., 2018). Moreover, chimeric Cas9 produced through ortholog analysis and Cas12a (Cpf1) variant produced through structure-guided mutagenesis show the effect of PAM sequence expansion and off-target effect reduction (Kleinstiver et al., 2019; Ma et al., 2019).

Recently, RNA-guided large DNA insertion tools were developed by combining the DNA integration capability of transposases and the function of target recognition of CRISPR-Cas. Insertion of Transposable Elements by Guide RNA-Assisted Targeting (INTEGRATE) and CRISPR-associated transposase from cyanobacteria *Scytonema hofmanni* (ShCAST) can efficiently integrate DNA segments into the genome of *E. coli* (Klompe et al., 2019; Strecker et al., 2019). These technologies can insert desired genes and pathways into the genome, and thus can be used to synthesize biological systems with intended functions.

Single Nucleotide Editing

Genome editing mediated by the CRISPR-Cas system requires cleavage of two strands of a target DNA and recombination (Hsu et al., 2014; Knott & Doudna, 2018). Since double-strand breaks of a target DNA can occur even if a mismatch exists between the edited target DNA and the guide RNA, genome editing at the single nucleotide level is hardly achieved even in microbial cells with low genome complexity (Lee et al., 2020a). Several CRISPR-Cas genome editing technologies, such as base editor and prime editor, have been developed to bypass the off-target effect without double-strand breaks (Fig. 1).

A base editor (BE), made through the fusion of dCpf1, dCas9, and nCas9 with a base deaminase, introduces point mutations into the target DNA without DSB (Gaudelli et al., 2017; Grunewald et al., 2019; Li et al., 2018d). An adenine base editor (ABE) facilitates the conversion of A:T to G:C, and cytidine base editor (CBE) converts C:G to T:A base pairs (Gaudelli et al., 2017; Komor et al., 2016). For example, in *S. aureus*, ABE converts A to G in 4–8 editing windows with > 50% editing efficiency (Zhang et al., 2020). In *galK* of *E. coli*, CBE achieves point mutagenesis with an efficiency of 61%–95%, and C to T substitution is mainly induced in 17–20 bases in the upstream region of the PAM sequence (Banno et al., 2018). ABE and CBE have been applied to various organisms, including eukaryotes and some bacteria, to introduce transition point mutations (Chen et al., 2018b; Luo et al., 2020; Wang et al., 2018d). A recently developed glycosylase base editor (GBE) can mediate base transversion such as C to A and C to G. It is composed of nCas9, a cytidine deaminase, and an uracil-DNA glycosylase (Ung). It converts C to A with an average editing specificity of 93.8% in *E. coli* (Zhao et al., 2021). However, if these BEs are adjacent to the same base, an unwanted bystander editing effect may occur (Lee et al., 2020b).

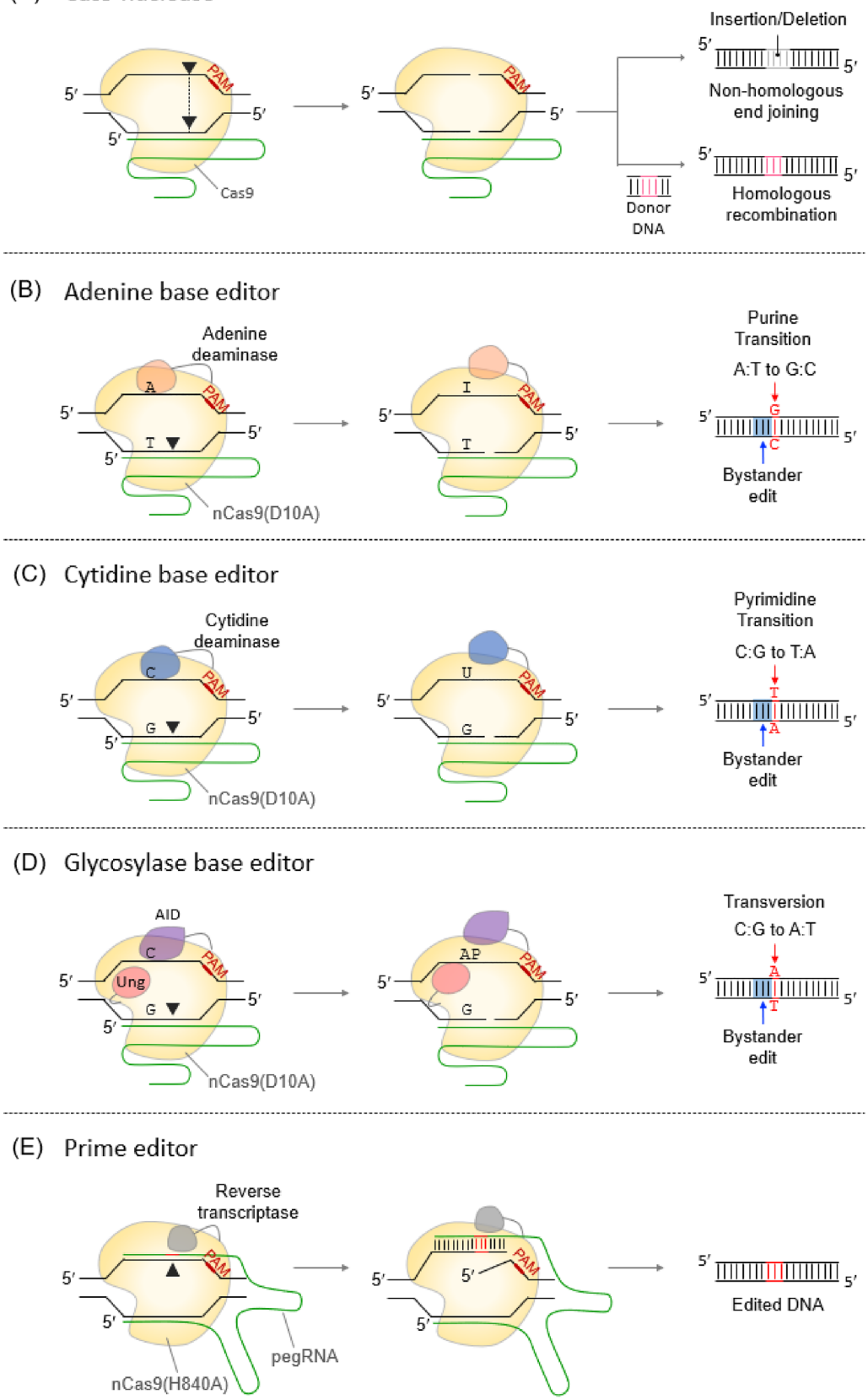
CasMINI, half the size of Cas9 and Cas12, was engineered from natural type V-F Cas12f (Cas14) system by gRNA and protein engineering (Xu et al., 2021). Deactivated CasMINI-mediated adenine base editor (dCasMINI-ABE) showed the most efficient A to G conversion in a narrow window (3–4 bp downstream region from the PAM). TnpB-based ABE, made through the fusion of the C-terminus of dTnpB with a modified dimer of TadA adenosine deaminase, facilitates A to G conversion in the PAM-proximal region (Kim et al., 2022). The conversion efficiency of TnpB-based ABE was higher than that of Cas12f-based ABE, but still lower than that of SpCas9-based ABE.

A prime editor (PE) uses Cas9 nickase fused with reverse transcriptase and prime editing sgRNA (pegRNA). It can edit the genome through various processes, such as insertion, deletion, and point mutation, as programmed in the pegRNA sequence (Anzalone et al., 2019). PE performs 2–3 bp substitution, insertion, and deletion in the chromosomal DNA of *E. coli* with an efficiency of 26% (Tong et al., 2021). However, loading a PE system is slightly difficult in a size-limited vector because of the large size of the construct consisting of an nCas protein, a reverse transcriptase, and pegRNA (Arroyo-Olarte et al., 2021).

Accurate genome editing with Cas dsDNA nuclease and target-mismatched and truncated guide RNAs has also been developed (Lee & Lee, 2021) (Fig. 2). Target-mismatched guide RNAs of Cas9 and Cpf1 can discriminate between a single-nucleotide-edited target and an unedited target and efficiently edit a single nucleotide in the genomes of *E. coli* and *Corynebacterium glutamicum*, respectively (Kim et al., 2020c; Lee et al., 2020a). Furthermore, a single-nucleotide in the cI^{857} repressor gene of the bacteriophage λ genome was accurately corrected by the aid of target-mismatched sgRNA and Cas9 complex, which restored thermostable λ lysogenic *E. coli* cells (Lee et al., 2022a). Cas9 with 5′-truncated sgRNA and Cpf1 with 3′-truncated crRNA improve on-target specificity and reduce off-target effect (Fu et al., 2014; Kim et al., 2017). Besides, 5′-truncation of sgRNA in Cas9 and 3′-truncation of crRNA in Cpf1 greatly enhance the efficiency of oligonucleotide-directed single nucleotide editing in the microbial genome (Lee et al., 2021, 2022b).

Nucleic Acid Diagnostics

The sequence of nucleic acids characteristic of each disease-causing virus or microbial pathogen can be used as a diagnostic biomarker. The CRISPR-Cas complex can recognize and cut the target nucleic acid, thereby indicating


(A) Cas9 nuclease
Insertion/Deletion
Non-homologous end joining
Homologous recombination
Donor DNA
Cas9
PAM
(B) Adenine base editor
Adenine deaminase
Purine Transition
A:T to G:C
Bystander edit
nCas9(D10A)
(C) Cytidine base editor
Cytidine deaminase
Pyrimidine Transition
C:G to T:A
Bystander edit
nCas9(D10A)
(D) Glycosylase base editor
AID
Ung
AP
Transversion
C:G to A:T
Bystander edit
nCas9(D10A)
(E) Prime editor
Reverse transcriptase
pegRNA
nCas9(H840A)
Edited DNA

◂**Fig. 1** CRISPR-Cas-based genome editing tools. **A** Cas9 nucleases create double-strand breaks in a target DNA. Indel and subsequent nonhomologous end joining inactivate the target gene. If a donor DNA is added, the target gene can be edited to the desired sequence. Filled triangles indicate cleavage sites. **B** Adenine base editors (ABEs), composed of an nCas9 fused with an adenine deaminase, mediate A-to-G transition. Adenine deaminase catalyzes the deamination of A to hypoxanthine (I), which is recognized as G, forming a G:C base pair. **C** Cytosine base editors (CBEs) convert C:G into T:A base pairs by using cytidine deaminase. **D** Glycosylase base editors (GBEs) mediate C-to-A transversion by using an nCas9 (D10A) fused with activation-induced cytidine deaminase (AID) and uracil DNA glycosylase (Ung). AID-nCas9-Ung binds to the target DNA and creates a nick. AID cleaves the amine group at C to form U. Ung excises the U base, forming an apurinic/apyrimidinic (AP) site that initiates DNA repair. **E** Prime editors are composed of nCas9, reverse transcriptase (RT), and prime editing guide RNA (pegRNA). nCas9 (H840A) cleaves the target DNA. The RT polymerizes a new DNA strand complementary to the pegRNA sequence on the nicked strand

the presence of a nucleic acid of a specific sequence in the sample (Fig. 3). Among various Cas nucleases, Cas9, Cas12, and Cas13, which belong to Class 2 whose effector complex is a single polypeptide, are mainly used to develop nucleic acid sensors (Liu et al., 2022c).

CRISPR-based diagnosis has the advantage of not requiring a sequencing step of target nucleic acids. However, since a sufficient amount of nucleic acids is necessary to detect a signal, DNA or RNA should be amplified. For rapid diagnosis in a single tube without equipment, DNA is amplified using isothermal reactions such as recombinase polymerase amplification (RPA) and loop-mediated isothermal amplification (LAMP).

Target Cleavage and Detection

When a specific DNA sequence is recognized as a target by the Cas9-sgRNA complex, a cleaved double-stranded target DNA is retained and can be detected in various ways (Strich & Chertow, 2019). For example, a toehold switch method has been proposed to identify Zika virus strains with CRISPR-Cas9 by designing strain variant sequences with PAM sequences. If variation exists in the viral DNA sequence, the Cas9/sgRNA complex is unable to cleave the target DNA; subsequently, the full-length trigger mRNA is transcribed to activate the toehold switch (Pardee et al., 2016). Bacterial antibiotic resistance genes are detected by designing a fluorescent probe to bind to the strand cut via the Cas9-sgRNA complex (Muller et al., 2016). Moreover, a sensitive DNA detection method is developed to detect isothermally amplified DNA by using the DNA cut by Cas9-sgRNA as a primer (Huang et al., 2018). Similarly, DNA sequence variation can be detected by inducing the target cleavage by Cas9/sgRNA in two different positions, thereby producing universal primer binding sequences at the 5′-ends of the cleaved strands; this variation can also be observed by performing qPCR (Gao et al., 2021). In addition, FnCas9 Editor Linked Uniform Detection Assay (FELUDA) was developed to detect single nucleotide variants of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Azhar et al., 2021).

With the development of deactivated Cas9 (dCas9) in which the nucleolytic activity of the RuvC and HNH nuclease domains of Cas9 has been removed (Qi et al., 2013), a diagnostic method for detecting the binding of the dCas9/sgRNA complex to the target DNA has been studied. When the dCas9/sgRNA complex binds to two different targets, the association of the partial luciferase fragments fused with each dCas9 can form an active full-length luciferase, which produces the luminescence (Zhang et al., 2017b). For miRNA detection, rolling circle amplification and dCas9-fused split-horseradish peroxidase techniques can be used (Qiu et al., 2018). Methicillin-resistant *Staphylococcus aureus* (MRSA) can be detected through fluorescence in situ hybridization (FISH) by connecting a magnetic bead to the dCas9 protein (Guk et al., 2017). In addition, a diagnostic method for detecting the change in the ionic current rectification caused by the binding of dCas9/sgRNA to a target in an aluminum-based sensing chip has been reported (Sun et al., 2022).

Trans-Cleavage Activity

The nuclease activity of Cas protein is activated through the binding of the gRNA/Cas complex to the target nucleic acid (Chen et al., 2018a; Lim et al., 2016). Unlike Type II Cas9, whose nuclease activity is lost upon target cleavage, Type V and Type VI Cas nucleases form a complex with a guide RNA and exhibit a nonspecific cleavage activity on ssDNA present in the vicinity even after the target DNA/RNA is cleaved, which is called *trans*-cleavage (Yuan et al., 2020). Based on this phenomenon, a diagnostic method has been developed to indirectly measure the *trans*-cleavage activity of Cas12 or Cas13 by fluorescence (Wang et al., 2020b), color development (Wang et al., 2022a), potential difference (Hajian et al., 2019), or other techniques.

Unlike Cas9, whose PAM sequence is located at the 3′ end of the target, Cas12 mainly has a T-rich PAM located at the 5′ end of the target and recognizes dsDNA and ssDNA as targets (Zetsche et al., 2015). The one-HOur Low-cost Multipurpose highly Efficient System (HOLMES) was

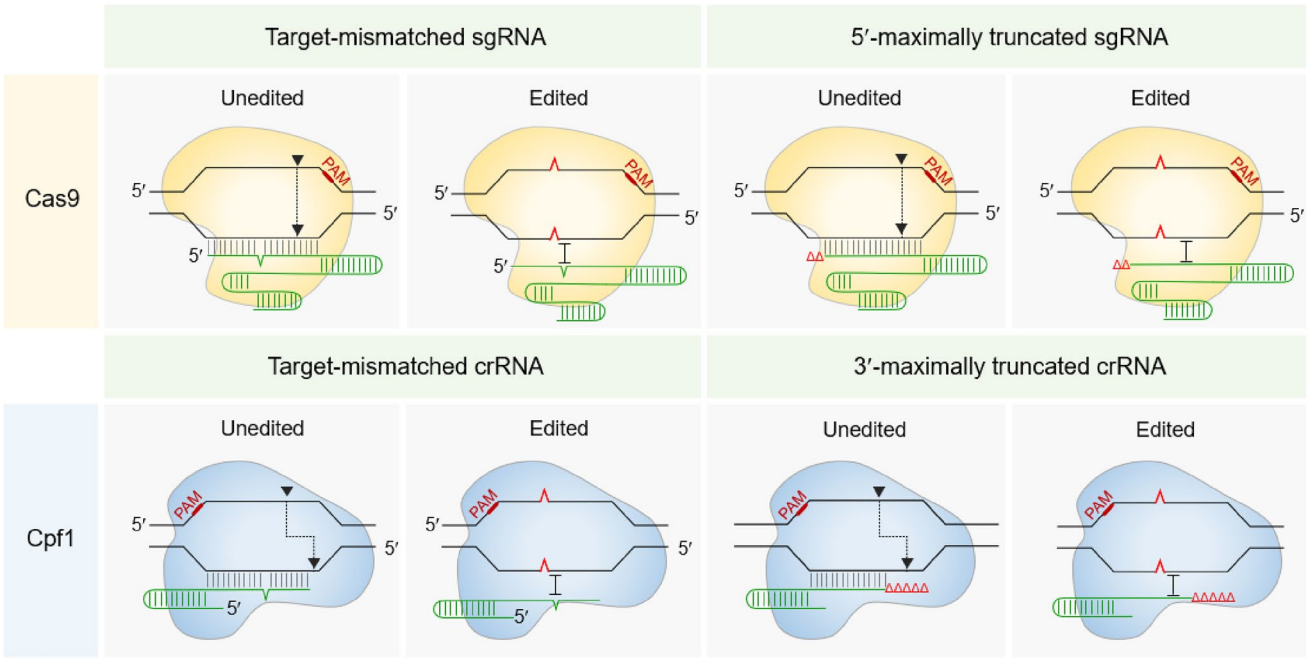


Fig. 2 Accurate genome editing with target-mismatched or maximally truncated guide RNAs in CRISPR-Cas systems. Unedited target DNAs are cleaved by Cas nucleases (Cas9 or Cpf1) with target-mismatched or maximally truncated guide RNAs (sgRNA for Cas9, and crRNA for Cpf1). Single base/nucleotide edited targets are not cleaved by Cas nucleases with target-mismatched or maximally truncated guide RNAs. The intolerance of single-base/nucleotide mismatch between the edited target DNA and target-mismatched or maximally truncated guide RNAs enables single base/nucleotide genome editing

developed as a diagnostic method that can detect single nucleotide variation by amplifying DNA/RNA in a sample via (RT-)PCR and optimizing crRNA that forms a complex with LbCas12a (Li et al., 2018c). In HOLMESv2 with AacCas12b, a diagnostic method has been constructed for detecting dsDNA, and ssDNA through (RT-)LAMP and asymmetric PCR (Li et al., 2019a). It can also distinguish a single nucleotide polymorphism (SNP) locus in the target DNA of the human genome. Through this method, SARS-CoV-2 variant (Najjar et al., 2022; Rossetti et al., 2022; Wu et al., 2022) and African swine fever virus (Qin et al., 2022) are diagnosed.

Unlike HOLMES, DNA Endonuclease-Targeted CRISPR Trans Reporter (DETECTR) is a diagnostic method to distinguish between different HPV strains by isothermally amplifying target DNA in the sample with RPA and then detecting the fluorescent signal of the ssDNA-FQ reporter generated by *trans*-cleavage following the cleavage of the dsDNA target of Cas12a (Chen et al., 2018a).

With this method, SNPs between *Bacillus anthracis* and *B. cereus*, have been distinguished, and specific species have been identified (Wang et al., 2022a). In addition, various types of pathogens, including MRSA (Wang et al., 2022b), *Mycoplasma pneumoniae* (Deng et al., 2022), and SARS-CoV-2 (Sun et al., 2021), have been diagnosed. All-In-One-Dual CRISPR-Cas12a (AIOD-CRISPR) is a method that can detect nucleic acids in a single reaction system without a separate pre-amplification step (Ding et al., 2020). It can sensitively detect the nucleic acids of SARS-CoV-2 and human immunodeficiency virus (HIV) by utilizing dual crRNAs.

Unlike Type II Cas9 and Type V Cas12 that cut DNA targets, Type VI Cas13 nucleases recognize and cut RNA as a target (Abudayyeh et al., 2016). Because of a higher turnover, *trans*-cleavage of Cas13a occurs relatively faster than that of Cas12a (Nalefski et al., 2021). Therefore, with the advantage of the fast detection of fluorescence signals, after the DNA target is converted to RNA, Cas13 is used for diagnosis. Specific high-sensitivity enzymatic reporter unlocking (SHERLOCK) amplifies DNA from a target DNA/RNA via (RT-)RPA and produces target RNA through in vitro transcription. Then, the RNA probe is cleaved via Cas13a *trans*-cleavage, which occurs in the presence of the target RNA, to generate a fluorescence signal (Gootenberg et al., 2017).

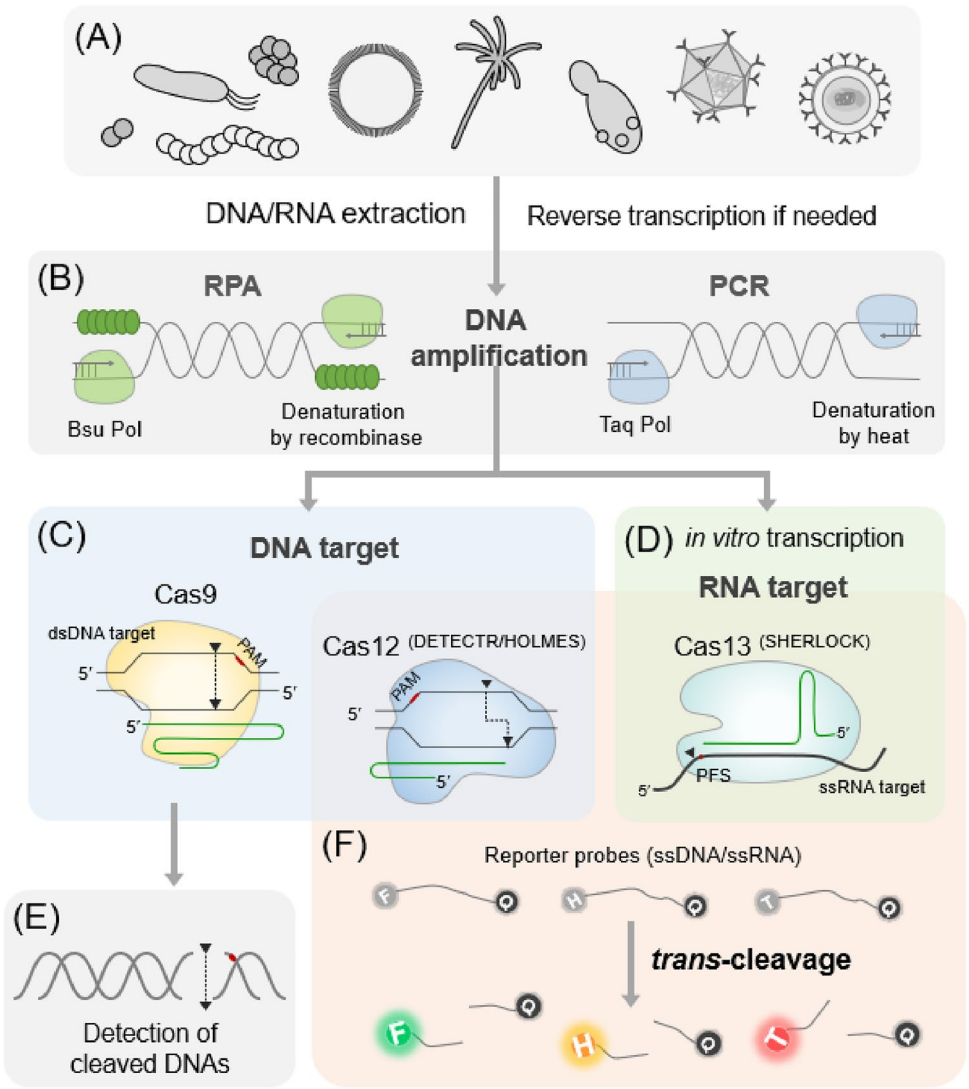


Fig. 3 Nucleic acid diagnostics via CRISPR-Cas systems. Schematic of the operating principle of a CRISPR-based diagnostic system is shown. **A** Depending on the target gene to be detected, DNA or RNA is extracted from pathogens such as viruses, bacteria, fungi, and yeasts. DNA is amplified via recombinase polymerase amplification (RPA) or PCR and used for nucleic acid detection. RNA can be reversely transcribed to cDNA, and subsequent RPA or PCR can amplify the cDNA. **B** In RPA, double-stranded DNA denatured by recombinase is isothermally amplified through the extension of the template DNA with Bsu DNA polymerase with a strand displacement activity. In PCR, double-stranded DNA is denatured by heat, and Taq DNA polymerase with high thermal stability is used for DNA amplification. **C** Cas9 or Cas12 and the corresponding guide RNA complex recognizes and cleaves the amplified DNA. **D** For RNA-targeting CRISPR enzymes, including Cas13, the amplified DNA is transcribed into RNA via in vitro transcription. **E** Cas9-based diagnosis is performed by detecting the cleaved target DNA itself. **F** Cas12- and Cas13-based diagnoses are conducted by detecting signals released when a single-stranded DNA or RNA reporter probe is cleaved by *trans*-cleavage. Each letter in a circle indicates the following: F, carboxyfluorescein (green); H, hexachlorofluorescein (orange); T, Texas Red; and Q, Quencher

SHERLOCK diagnosis is mainly used to detect a pseudovirus in swab or food (Wang et al., 2021) or to detect RNA viruses such as feline calicivirus (Huang et al., 2022) or SARS-CoV-2 (Casati et al., 2022). SHERLOCK can also detect cancer mutations in cell-free DNA, and SNPs from human saliva (Gootenberg et al., 2017). Heating unextracted diagnostic samples to obliterate nucleases (HUDSON), a thermal/chemical processing method of collected samples, has been combined with SHERLOCK to enable faster and more accurate diagnosis and analysis (Myhrvold et al.,

Table 2 CRISPR-based diagnosis in microbial pathogens

Cas effector	Target amplification	Target pathogen	Sample type	Limit of Detection	Chromophores/ fluorophores	Time	Description	References
Cas9	Reverse transcription, NASBA, in vitro transcription (T7)	ZIKV	Synthetic viral vector	1 fM	Colorimetry (Toehold switch)	3 h	Toehold inactivation by truncated RNA result from Cas9 cleavage	Pardee et al. (2016)
	Reverse transcription, EXPAR	*Listeria monocytogenes*	Total RNA	0.82 amole	Fluorescence (SYBR Green I)	1 h	ssDNA primer generation by Cas9 cleavage	Huang et al. (2018)
	PCR, RPA	*L. monocytogenes*	gDNA	150 copies	Colorimetry (AuNP)	1 h	Probe hybridization by Cas9 cleavage	Wang et al. (2020a)
	LAMP	*Salmonella*, *Neisseria meningitidis*	gDNA	80 copies	Fluorescence (SYBR Green I)	1 h	Removal of contaminant by Cas9 cleavage	Bao et al. (2020)
	Reverse transcription, PCR, RPA	SARS-CoV-2	gDNA	10 copies	Fluorescence (FAM)	1 h	FELUDA	Azhar et al. (2021)
dCas9	PCR	*M. tuberculosis*	gDNA	50 pM*	Luminescence (Firefly luciferase)	2 h	Partial protein hybridization by dCas9 binding	Zhang et al. (2017b)
	-	Methicillin-resistant *Staphylococcus aureus* (MRSA)	Bacterial cell lysate	10 CFU/ml	Fluorescence (SYBR Green I)	3 h	Captured with His-tagged dCas9, Ni–NTA column	Guk et al. (2017)
	RCA	–	Synthetic miRNA	35.4 aM	Colorimetry (TMB)	4 h	TMB oxidation and color development by full-length horseradish peroxidase	Qiu et al. (2018)
Cas12a	PCR	Pseudorabies virus (PRV), Japanese encephalitis virus (JEV)	gDNA	0.5 aM	Fluorescence (HEX)	1 h	HOLMES (truncated crRNA)	Li et al. (2018c)
	RPA	Human papillomavirus (HPV)	Synthetic viral vector	1 aM	Fluorescence (FAM)	2 h	DETECTR	Chen et al. (2018a)
	RPA	*Mycoplasma*	Synthetic vector	10 aM	Fluorescence (FAM)	0.5 h	One-pot reaction	Wang et al. (2019)
	RPA	*E. coli* O157:H7, *S. aureus*	gDNA	1 CFU/ml	Fluorescence (HEX)	1 h	One-pot reaction	Wang et al. (2020b)
	RPA	*B. anthracis*	Bacterial Plasmid	1 copy/rxn	Colorimetry (ABTS)	1.5 h	Visible to the unaided eye	Wang et al. (2022a)
	RAA (similar to the RPA)	*P. aeruginosa*	Bacterial cell culture	1 aM, 10^3 CFU/ml	Fluorescence (FAM)	4 h	Centrifugal microfluidic chip	Chen et al. (2020)
	PCR	*Shigella dysenteriae*	Bacterial cell culture	10 aM	Fluorescence (FAM)	2 h	Biotinylated ssDNA reporter + gold nanoparticle	Sun et al. (2020)
	RT-RPA	SARS-CoV-2	Pseudoviruses	1 copy/μl	Fluorescence (FAM)	1 h	One-pot reaction	Sun et al. (2021)

Table 2 (continued)

Cas effector	Target amplification	Target pathogen	Sample type	Limit of Detection	Chromophores/ fluorophores	Time	Description	References
	RPA	SARS-CoV-2, HIV-1	Synthetic viral vector	1.3 copies, 1.2 copies	Fluorescence (FAM)	40 min	AIOD-CRISPR (One-pot reaction)	Ding et al. (2020)
Cas12b	LAMP, Asymmetric PCR	JEV	Virus	10 aM	Fluorescence (FAM)	1 h	HOLMESv2 (Cas12b)	Li et al. (2019a)
Cas13a	RPA, in vitro transcription (T7)	*P. aeruginosa*, ZIKV, Dengue virus (DENV)	gDNA, Synthetic viral vector	2 aM	Fluorescence (FAM)	2 h	SHERLOCK	Gootenberg et al. (2017)
	RPA, in vitro transcription (T7)	ZIKV, DENV	Clinical sample	20 aM	Fluorescence (FAM)	2 h	HUDSON (sample treatment)	Myhrvold et al. (2018)
	PCR, in vitro transcription (T7)	*S. aureus*	Food sample	1 CFU/mL	Fluorescence (FAM)	4 h	Cas13a has stronger *trans*-cleavage activity than Cas12	Zhou et al. (2020)
	in vitro transcription (T7)	SARS-CoV-2	Synthetic viral vector	82 copies	Fluorescence (Broccoli)	1 h	RNA fluorescence using light-up aptamers	Wang et al. (2021)
	PCR, in vitro transcription (T7)	113 pathogenic strains	gDNA	1 copy	Fluorescence	3 h	One-pot amplification of over 50 targets	Thakku et al. (2022)
	RPA, in vitro transcription (T7)	SARS-CoV-2 variants	Clinical sample	2.5 copies/µl	Fluorescence (TEX 615)	1 h	Sensitive detection of Delta and Omicron variants	Casati et al. (2022)
	Isothermal amplification, in vitro transcription (T7)	*S. enterica* Enteritidis	gDNA	1 copy	Fluorescence (FAM)	2.5 h	Probe contains aptamer domain as identification element	Shen et al. (2020)
	RPA, in vitro transcription (T7)	*P. aeruginosa*, *S. aureus*, ZIKV, DENV	gDNA, Total RNA	8 zM	Fluorescence (FAM)	1 h	SHERLOCKv2	Gootenberg et al. (2018)
	Reverse transcription, PCR, in vitro transcription	SARS-CoV-2	Total RNA	10 copies/µl	Fluorescence (FAM)	3 h	CREST (SARS-CoV-2 RNA detection method)	Rauch et al. (2021)

ABTS 2,2′-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, *AIOD-CRISPR* All-in-One Dual CRISPR-Cas12a, *AuNP* Gold Nanoparticle, *CREST* Cas13-based, Rugged, Equitable, Scalable Testing, *DENV* Dengue virus, *DETECTR* DNA Endonuclease-Targeted CRISPR Trans Reporter, *EXPAR* Exponential Amplification Reaction, *FAM* Fluorescein amidite, *FELUDA* FnCas9 Editor-Limited Uniform Detection Assay, *gDNA* Genomic DNA, *HEX* Hexachloro-fluorescein, *His-tagged* Polyhistidine-tagged, *HIV-1* Human immunodeficiency virus type 1, *HOLMES* One-hour Low-cost Multipurpose Highly Efficient System, *HPV* Human papillomavirus, *HUDSON* Heating Unextracted Diagnostic Samples to Obliterate Nucleases, *JEV* Japanese encephalitis virus, *LAMP* Loop-mediated isothermal amplification, *miRNA* microRNA, *MRSA* Methicillin-resistant *Staphylococcus aureus*, *NASBA* Nucleic Acid Sequenced Based Amplification, *Ni–NTA* Nickel-nitrilotriacetic acid, *PCR* Polymerase chain reaction, *PRV* Pseudorabies virus, *RAA* Recombinase-aided amplification, *RCA* Rolling circle amplification, *RPA* Recombinase polymerase amplification, *RT* Reverse transcription, *SARS-CoV-2* Severe acute respiratory syndrome coronavirus 2, *TEX* Texas Red, *TMB* 3,3′,5,5′-Tetramethylbenzidine, *ZIKV* Zika virus

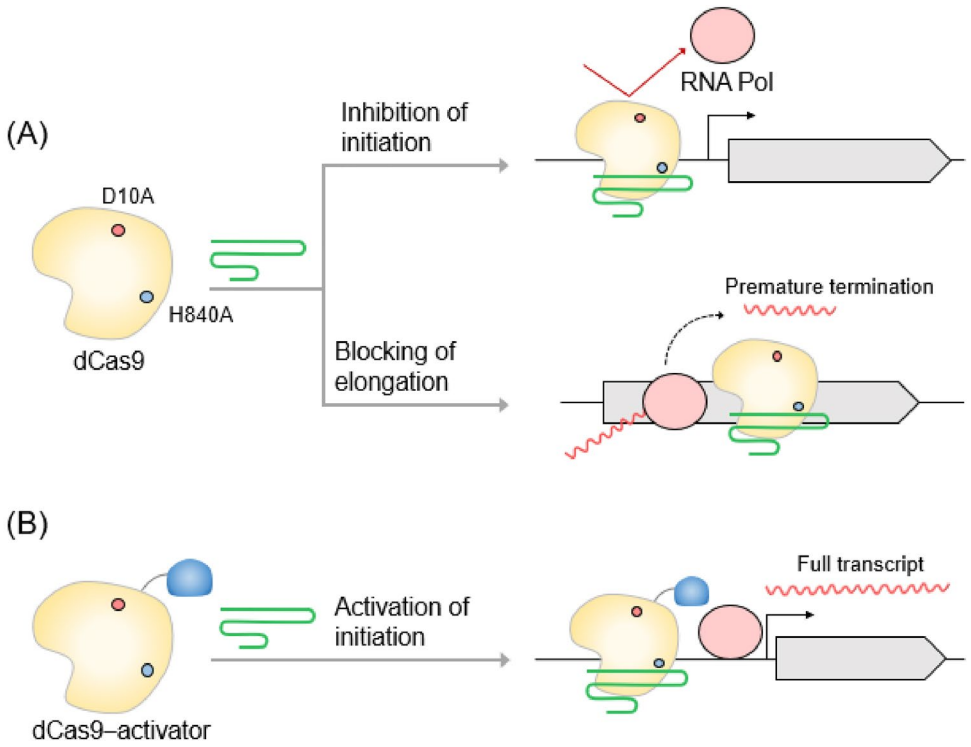


Fig. 4 Transcriptional regulation by deactivated Cas proteins. **A** CRISPR interference (CRISPRi). Deactivated Cas9 (dCas9) enables the regulation of gene expression by blocking the transcriptional initiation or elongation of RNA polymerase (RNA Pol). **B** CRISPR activation (CRISPRa). dCas9 fused with a transcription activator binds upstream of the target promoter to recruit RNA Pol and activates the transcription of the target gene

2018). This platform can distinguish four dengue virus (DENV) serotypes, and detect region-specific SNPs in zika virus (ZIKV) samples (Chertow, 2018). SHERLOCKv2 has also been developed to detect DNA at a concentration of 8 zM through the amplification of a fluorescence signal by using synergistically activated Csm and Cas13a (Gootenberg et al., 2018).

Cas13a-Based, Rugged, Equitable, and Scalable Testing (CREST) was developed to address major hurdles in limiting scalability of RT-qPCR method detecting SARS-CoV-2 using widely available enzymes, fluorescent visualizers, and portable thermocyclers. CREST has been shown to have sensitivity comparable to that of RT-qPCR in COVID-19 test (Rauch et al., 2021). Microfluidic Combinatorial Arrayed Reactions for Multiplexed Evaluation of Nucleic acids (mCARMEN) is a method that can detect multiple viruses and mutants simultaneously. Six SARS-CoV-2 variant lineages, including Delta and Omicron, can be identified by using 26 crRNA pairs, individually or in combination (Welch et al., 2022). Multiple samples can be examined and detected using four Cas nucleases with different types of single-stranded nucleic acid probes. Other representative cases of Cas nuclease diagnosis are summarized in Table 2.

In vivo CRISPR Regulation

CRISPR-Cas evolved into deactivated Cas9 (dCas9) that can regulate gene transcription beyond genome editing. The CRISPR-dCas system has been expanded through fusion with various effector domains such as transcriptional repressors or activators (Farzadfard et al., 2013; Konermann et al., 2015). As shown in Fig. 4, the binding of the sgRNA-dCas protein complex to the target DNA can block the initiation and elongation of transcription by interfering with the binding of RNA polymerase or transcription factors (Qi et al., 2013) through a process called CRISPR interference (CRISPRi). The CRISPRi system can reversibly inhibit the transcription of multiple target genes (Qi et al., 2013; Zhang et al., 2021). The combination of a DNase-dead Cpf1 mutant (ddCpf1), and a crRNA array is used to simultaneously inhibit the transcription of four genes (Zhang et al., 2017a). In yeast, various transcriptional repressors are fused with dCas protein to regulate transcription more effectively (Schwartz et al., 2017; Wensing et al., 2019).

CRISPR activation (CRISPRa) that can activate gene expression by promoting the recruitment of transcriptional activators to target DNA sequences has been developed (Bikard et al., 2013). Various transcriptional activators, including the ω subunit of RNA polymerase and phage activator AsiA, have been fused with dCas9 in a bacterial

Table 3 CRISPR-mediated gene regulation

Organism	dCas protein	Species	Effector domain	Target	Feature	References
Bacteria	dCas9	*Escherichia coli*	None	*mRFP*, *sfGFP*	Characterization of factors that affect silencing efficiency	Qi et al. (2013)
			PhlF	*rfp*	Construction of the nontoxic version of dCas9 by making R1335K mutation to dCas9 and fusing it to the PhlF repressor	Zhang and Voigt (2018)
			None	*gal* promoter, *galETK*	Repression of DNA targets by CRISPRi with expanded PAM sequences	Kim et al. (2020a)
		Corynebacterium glutamicum	None	*pyc*, *gltA*, *idsA*, *glgC*	Repression of single or double target genes by using two plasmid system (dCas9 plasmid, sgRNA plasmid)	Park et al. (2018b)
		Cyanobacterium Anabaena	None	*glnA*	Fine-tuning the GlnA protein by strictly regulating dCas9 expression via the *TetR* induction system	Higo et al. (2018)
		Mycobacterium tuberculosis	None	*dnaE1*, *pptT*	Achieved 20- to 100-fold knockdown of target genes using $dCas9_{Sth1}$ with minimal proteotoxicity	Rock et al. (2017)
	dCas9 – activator	*Bacillus subtilis*	ω subunit	*prsA*, *nprB*, *bpr*, *vpr*	260-fold enhancement of BLA production via the promoter engineering strategy OAPS	Lu et al. (2019)
		E. coli *Salmonella enterica* *Klebsiella oxytoca*	AsiA	*gfp*	Improving the potency of dCas9-AsiA using random PCR mutagenesis	Ho et al. (2020)
		Pseudomonas putida	MCP-SoxS	*gtpch*, *ptps*, *sr*, *mvaES*	Using a modified gRNA (scRNA) that recruits RNA-binding protein fused with an effector domain	Kiattisewee et al. (2021)
	dCpf1	*C.glutamicum*	None	*pck*, *hom*, *pgi*, *gltA*	Simultaneous repression of four target genes by a single crRNA array	Li et al. (2020)
		E. coli	None	*malT*, *prop*, *degP*, *rseA*	Multiplex gene regulation using ddCpf1 with the remaining RNase activity	Zhang et al. (2017a)
	dCpf1 – activator	*B. subtilis*	RemA	*bdhA*, *acoA*, *ldh*, *pta*, *alsR*	Construction of CRISPR-assisted multiple genes editing and regulation system and crRNA array using SOMACA method	Wu et al. (2020)
		B. amyloliquefaciens	ω subunit	*secE*, *secDF*, *prsA*	Simultaneous activation of the expression of three genes by designing a crRNA array	Xin et al. (2022)
	dxCas9 – activator	*E. coli*	PspF	*sfGFP*, *mCherry*, *mTagBFP2*, *vioADC*	Using scRNA, which contained BoxB aptamers that bind to λN22plus peptide fused with activation domain	Liu et al. (2019b)
Fungi	dCas9 – activator	*Aspergillus niger*	p300	*breF*, *fuml*, *fwnA*	Regulation of the expression of secondary metabolic genes via dCas9-based histone modification	Li et al. (2021)

Table 3 (continued)

Organism	dCas protein	Species	Effector domain	Target	Feature	References
		Penicillium rubens	VPR	*DsRed*, *macR*	Using an AMA1 shuttle vector with a ribozyme-based sgRNA "plug-and-play" module	Mozsik et al. (2021)
		A. nidulans	VPR	*mdp cluster*, *AN8504 cluster*	Optimizing sgRNA positioning by creating genome-wide nucleosome maps	Schuller et al. (2020)
Yeast	dCas9	*Yarrowia lipolytica*	Mxi1	*ku70*, *ku80*	Achieving high HR rates by enhancing repression with Mxi1 fused with dCas9	Schwartz et al. (2017)
		Schizosaccharomyces pombe	None	*his2*$^{+}$, *his7*$^{+}$, *ade6*$^{+}$, *ura4*$^{+}$	Determination of the directionality of targeting sequences of sgRNAs	Ishikawa et al. (2021)
		Saccharomyces cerevisiae	Mxi1	*GFP*	Optimizing gRNA-promoter combinations by testing 101 gRNA structures on 14 promoters	Jensen et al. (2017)
		Candida albicans	Mxi1, Mig1	*ADE2*, *HSP90*	First CRISPRi system for use in *C. albicans*	Wensing et al. (2019)
	dCas9 – activator	*S. cerevisiae*	VP64	*GFP*	By targeting different sites, single crisprTF can be programmed to act as an activator and repressor	Farzadfard et al. (2013)
		Ogataea thermomethanolica	VP64	Promoter sequence of *SOD1*, *VPS1*, *YPT7*	Evaluation of the role of genes on heterologous protein secretion	Kruasuwan et al. (2021)
	dCpf1	*Pichia pastoris*	Mit1	*eGFP*	Construction of SynPic-X, a synthetic expression platform with iTSAD	Liu et al. (2022b)
		Y. lipolytica	KRAB	*gfp*, *vioABE*	Using multiplex gRNA strategy based on one-step golden-brick assembly technology	Zhang et al. (2018)
	dCpf1 – activator	*S. cerevisiae*	VP64 VP64-p65AD VP64-VPR	*CYC1p*, *RNR2p*	Designing a combinational metabolic engineering strategy based on an orthogonal tri-functional CRISPR system	Lian et al. (2017)

OAPS Oligonucleotide Annealing-based Promoter Shuffling, *scRNA* scaffold RNA, *ddCpf1* DNase-dead Cpf1, *SOMACA* Synthetic oligonucleotide-mediated assembly method, *VPR* VP64-p65-Rta, *iTSAD* improved transcriptional signal amplification device

system (Bikard et al., 2013; Ho et al., 2020). In *S. cerevisiae*, dCas9 fused with VP64 significantly enhances the activity of a reporter gene when sgRNA is located upstream of the TATA box (Farzadfard et al., 2013). CRISPRi/a has been used as a molecular tool for industrially important host gene expression regulation and gene function identification because of its easily programmable properties (Liu et al., 2017; Rousset et al., 2018). Representative studies on CRISPR-mediated microbial gene transcriptional regulation are summarized in Table 3.

Cellular System Optimization

Since the development of artificial gene circuits in 2000 (Elowitz & Leibler, 2000; Gardner et al., 2000), transcription factors (TFs) have been used to regulate gene expression in most synthetic circuits. TFs generally offer a high dynamic range, but their orthogonality, modularity, and programmability are limited; therefore, TFs are less ideal for synthetic biology (Zhang & Voigt, 2018). CRISPR-based gene circuits can be constructed to easily target and manipulate individual genes in complex regulatory networks within cells. In prokaryotes, CRISPR-based synthetic circuits mainly include logic circuits such as AND, NOR, and NIMPLY (Santos-Moreno & Schaerli, 2020). In *S. cerevisiae*, NOR gates are constructed via chromatin remodeler-combined CRISPRi, which allows minimal leak and digital responses (Gander et al., 2017).

The CRISPRi/a circuit can be linked to a cellular sensor system to control host metabolism in response to external stimuli (Mimee et al., 2015; Taketani et al., 2020). However, in the case of complex gene circuits, multiple gRNAs must share a limited intracellular pool of dCas9, which can reduce target gene repression (Li et al., 2018b). Therefore, a non-toxic version of $dCas9^{R1335K}$ with impaired ability to recognize PAM has been developed and fused with a PhlF inhibitor to solve this problem; in this way, the dCas9-based circuit design has been expanded in metabolic engineering and synthetic biology (Zhang & Voigt, 2018).

CRISPR-mediated gene regulation technology has been utilized to optimize the biosynthetic pathways of various metabolites in microorganisms and identify chemical–genetic interactions (Vanegas et al., 2017). CRISPRi has been mainly used to inhibit target genes, including essential genes, and direct carbon flux toward a desired product or bioactive compound (Kim et al., 2016b). In *Corynebacterium glutamicum*, the production of L-lysine and L-glutamate is improved by simultaneously inhibiting the expression of *pgi*, *pck*, and *pyk* via CRISPRi (Cleto et al., 2016). CRISPRa can be used to activate metabolic pathways related to the biosynthesis of a desired product. In *Pseudomonas putida*, mevalonate production was increased 40-fold through the activation of related genes via CRISPRa (Kiattisewee et al., 2021). In addition, CRISPR-mediated gene circuits or biosensors are used to alternately switch between cell growth and production phases and improve the production of desired metabolites (Shabestary et al., 2021). Furthermore, metabolite production can be further increased by combining CRISPRi/a technology with other metabolic engineering techniques, such as deletion, overexpression of specific genes, or optimization of growth media (Kozaeva et al., 2021; Lian et al., 2017).

Target Gene Screening

To adapt to various environments or conditions, cells are regulated by a complex network of numerous genes. CRISPR technology can be used for high-throughput genome-wide screening to achieve the desired cellular performance or obtain gene targets corresponding to phenotypes. Prior to CRISPR technology, RNAi-based screening has been widely used to identify genes involved in specific pathways, structures, or functions (Cronin et al., 2009). However, this approach has difficulties in finding the association between phenotype and gene knockdown because of the off-target effect on mRNA and incomplete gene suppression (So et al., 2019).

CRISPR-Cas screening via a gRNA library has been used to reveal genes, pathways, and mechanisms related to specific phenotypes or biological characteristics. CRISPR-Cas9 screening has also been used to identify genes associated with bacterial invasion (Pacheco et al., 2018) and resistance to antibiotics or chemicals (Garst et al., 2017). However, CRISPR knockout (CRISPR-KO) screening cannot reversibly regulate gene expression because it causes permanent gene disruption (So et al., 2019). CRISPRi/a, which can modulate gene expression levels and mediate reversible gene expression, has been developed and utilized for gene screening. CRISPRi screening is generally performed in two ways: pooled or arrayed (Bock et al., 2022).

In pooled CRISPR screening, a target gene is identified by sequencing the sgRNA extracted from cells showing a specific phenotypic change after a large amount of sgRNA library is introduced into them (Fig. 5). For example, essential auxotrophic and antibiotic resistance-related genes are screened using a sgRNA library (~60,000 sgRNAs) in *E. coli* (Wang et al., 2018b). CRISPRa in *S. cerevisiae* has

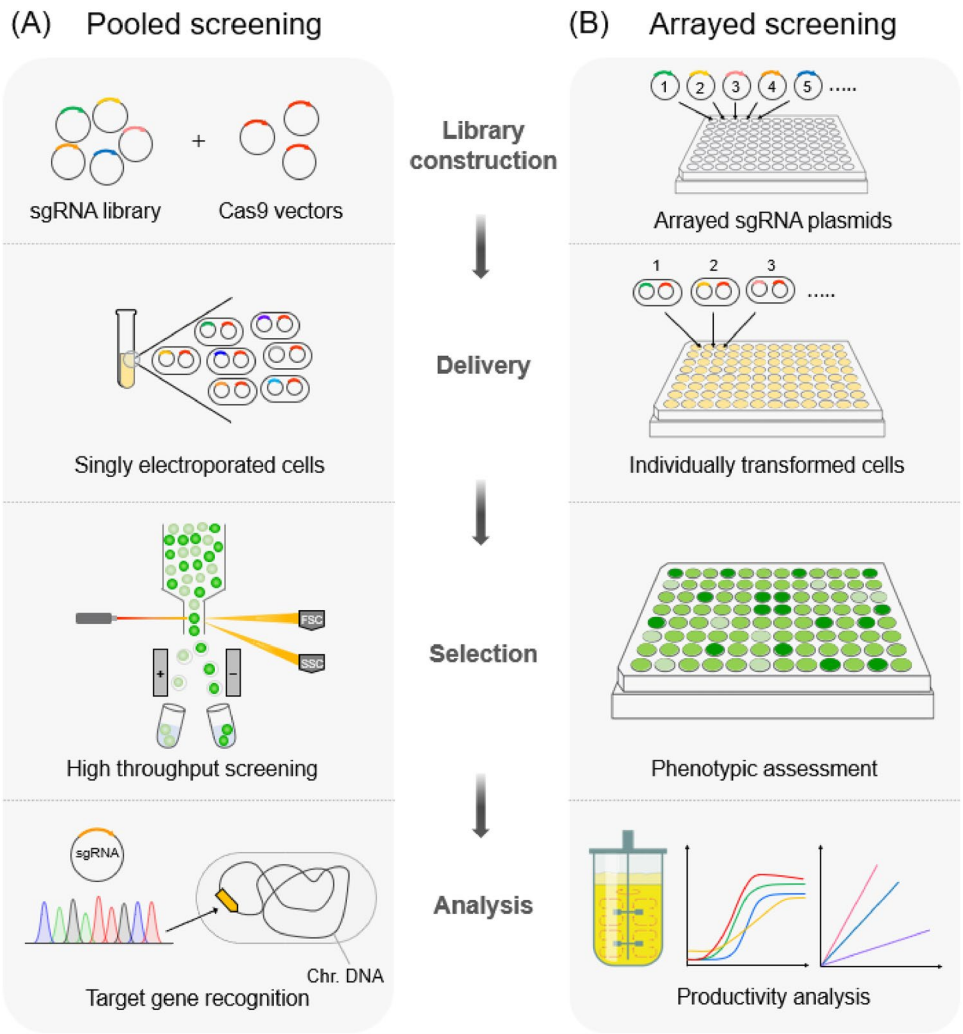


Fig. 5 Pooled and arrayed CRISPR screening methods. **A** In pooled screening, sgRNA library and Cas9 vector are delivered into cells within a single tube. Then, cells with specific phenotypes can be screened using high throughput facilities. Target genes are identified by DNA sequencing of sgRNAs extracted from the selected cells. **B** In arrayed screening, Cas9 vector and each sgRNA vector are delivered into individual cells. Cells with the desired phenotype are selected, and the productivity of the selected cells is analyzed

confirmed that OLE1 is important for the heat resistance of yeast (Li et al., 2019b).

In arrayed CRISPRi screening, one sgRNA targeting one gene per well is introduced into cells in a multi-well plate and the phenotype occurring in each cell is observed. For example, an L-proline exporter was discovered for L-proline hyperproduction by using a sgRNA library targeting the potential L-proline transporter genes in *Corynebacterium glutamicum* (Liu et al., 2022a). In addition, 28 phosphatase-encoding genes that increase terpenoid production have been identified in *E. coli* by using the CRISPRi system (Wang et al., 2018c). Thus, genome-wide CRISPRi/a screening is evolving into an effective synthetic biology tool that can help profile the relationship between phenotype and genotype and find targets for engineering in complex cellular networks.

Perspectives

CRISPR-Cas technology has been applied as a genome editing tool for various organisms, including prokaryotes and human cells (Jinek et al., 2012). Currently, highly specific and *trans*-cleaving nucleolytic activities of CRISPR-Cas are used for accurate genome editing (Lee & Lee, 2021) and diagnosis (Kaminski et al., 2021), respectively. Deactivated Cas protein is used not only to regulate the transcription of target genes but also to reveal the function of genes related to specific phenotypes through CRISPR screening.

In the future, CRISPR-based genome editing tools will be applied to various cells, from bacteria to humans via codon optimization and established genetic vectors. Changing the gut and soil microbiome through the production of customized strains will enable individual health management and improve crop yield and quality. In addition, CRISPR-Cas will be developed to innovate quick and easy biosensors that can detect pathogenic

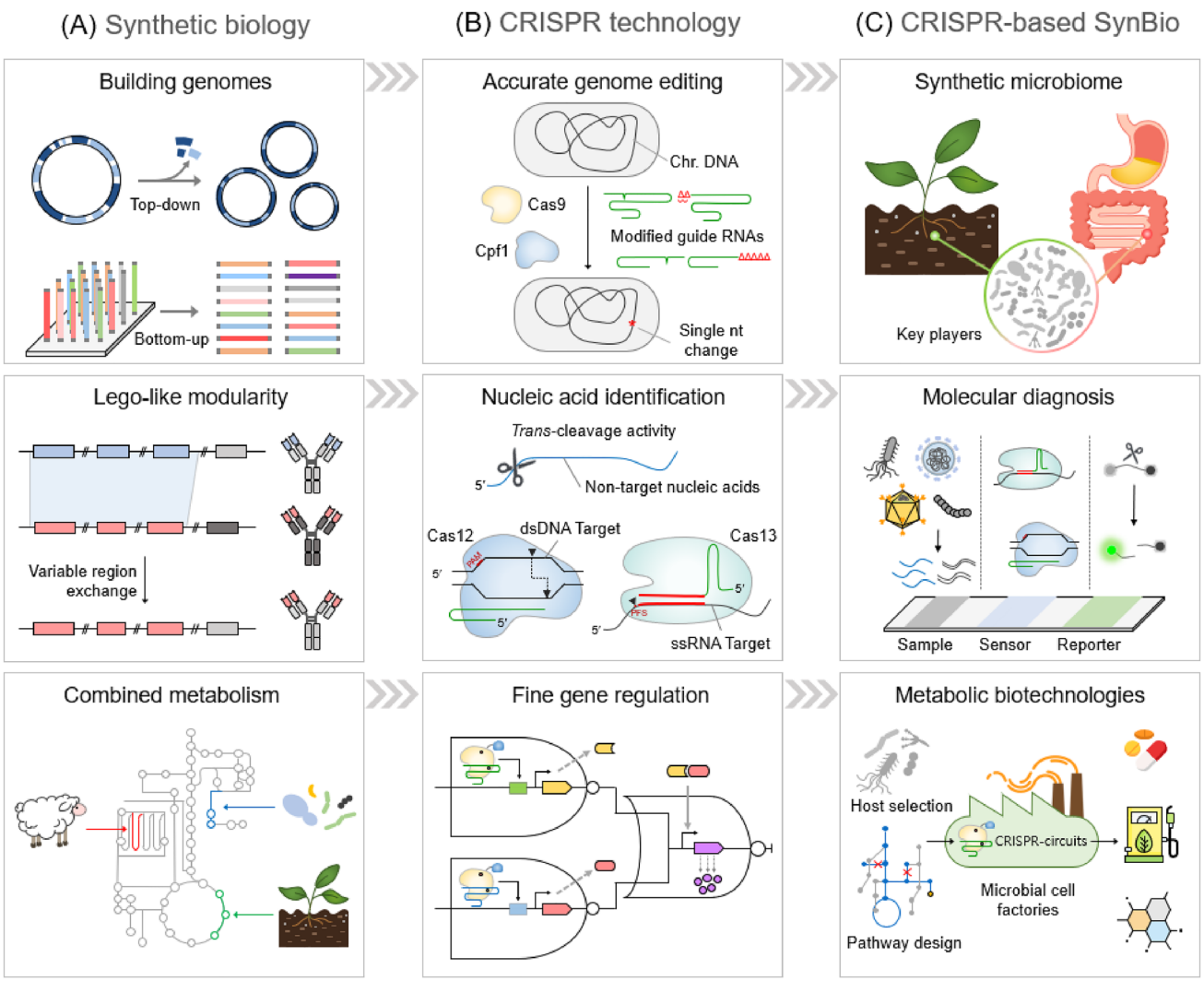


Fig. 6 CRISPR-Cas systems accelerate the development of synthetic biology. **A** Current synthetic biology can establish a new genome in top-down or bottom-up manners. Biomolecular engineering can be accelerated by Lego-like modularization. Combining the metabolic pathways of different origins can create pathways for the synthesis of new substances. **B** Development of various CRISPR technologies for accurate genome editing using modified guide RNAs, nucleic acid detection using *trans*-cleavage activity of Cas enzymes, and fine gene regulation by CRISPR-based artificial transcription factors. **C** Applications of microbial CRISPR technologies in synthetic biology: synthetic microbiome (e.g., regulation of plant/human-microbe interaction), molecular diagnosis (e.g., point-of-care testing to detect pathogens or genetic markers), and metabolic biotechnologies (e.g., production of pharmaceutical, bioenergy, and chemicals in microbial cell factories carrying CRISPR-based circuits)

microorganisms and genetic markers without using expensive equipment, which can greatly help in disease diagnosis and treatment. CRISPR-mediated gene regulation will improve the performance of industrial strains that produce useful biochemicals (Fig. 6).

Among various biotechnologies, CRISPR-Cas technology has become a key tool in the field of microbial metabolic engineering and synthetic biology by maximizing advantages such as ease of use and scalability of modular components. It has accelerated the design–build–test–learn cycle of synthetic biology to solve current problems such as healthcare, global epidemics, food shortages, and environmental pollution; thus, it will help achieve a sustainable future human life.

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Declarations

Conflict of Interest The authors declare that they have no conflict of interest.

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