

CRISPR-integrated Biosensors for Human Health: Novel strategies for pathogen detection and cancer screening

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CRISPR-integrated Biosensors for Human Health: Novel strategies for pathogen detection and cancer screening

Yawen He

Abstract (academic)

Rapid and accurate detection of pathogens and genetic mutations is essential for improving public health, guiding clinical decisions, and ensuring food safety. CRISPR-based biosensors offer a promising solution due to their programmability, sensitivity, and versatility. This dissertation focuses on the development of CRISPR-integrated biosensing platforms for assessing antimicrobial resistance, detecting bacterial pathogens, and identifying cancer-related genetic mutations.

Five diagnostic systems were developed using different CRISPR/Cas technologies. First, a CRISPR/Cas9-engineered T4 bacteriophage was used for rapid antimicrobial susceptibility testing in *Escherichia coli* (*E. coli*). Second, a CRISPR/Cas12a-based electrochemical sensor was designed for the detection of *Salmonella* Typhimurium in food samples. Third, a CRISPR/Cas14a system combined with blocker displacement amplification enabled sensitive detection of the BRAF V600E mutation in colorectal cancer cells. Fourth, a one-pot CRISPR/Cas13 biosensor was designed for simultaneous detection of *E. coli* O157:H7 serotype markers, validated in a mouse infection model. Finally, the sensitivity of the CRISPR/Cas12a system was enhanced through site-specific covalent crosslinking between Cas12a and crRNA, which improved detection of SARS-CoV-2 in clinical samples.

Together, these studies demonstrate the potential of CRISPR-based biosensors as powerful tools for diagnostics. The systems developed in this work are adaptable, sensitive, and suitable for applications in clinical diagnostics, food safety, and disease monitoring.

CRISPR-integrated Biosensors for Human Health: Novel strategies for pathogen detection and cancer screening

Yawen He

Abstract (general audience)

Early and accurate detection of disease is essential for protecting public health, guiding medical treatment, and ensuring food safety. This research focuses on developing easy-to-use, reliable diagnostic methods using CRISPR, a gene-targeting technology originally used for gene editing. By adapting CRISPR tools to recognize specific genetic material, these methods can identify bacteria, viruses, and even mutations linked to cancer with high precision.

Five different CRISPR-based methods were developed in this work. One method uses engineered viruses that infect bacteria to check if infections are resistant to antibiotics. When the bacteria survive in the presence of antibiotics, a color change occurs, offering a quick visual result. Another method targets *Salmonella* in food by producing an electrical signal when the bacteria are present. A third method detects a gene mutation often found in colorectal cancer, helping identify the disease at an early stage. A fourth method can find a notorious strain of *E. coli* by detecting two genetic markers in a single reaction. The final method improves the sensitivity of CRISPR tools, allowing them to detect SARS-CoV-2 viruses in clinic samples.

These diagnostic systems are designed to be fast, accurate, and suitable for use outside traditional laboratories. They can support early diagnosis, better monitoring of infectious diseases, and faster responses to outbreaks or public health threats. Overall, this research shows how CRISPR can be turned into a powerful tool for developing next-generation tests to detect pathogens and cancer-related changes in a simple and accessible way.

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List of Abbreviations

Abbreviation	Definition
AST	antimicrobial susceptibility testing
ATCC	american type culture collection
AuNPs	gold nanoparticles
BDA	blocker displacement amplification
Cas	CRISPR-associated
CDs	carbon dots
CFU	colony-forming unit
CPRG	chlorophenol red- β -D-galactopyranoside
CRISPR	clustered regularly interspaced short palindromic repeats
crRNA	CRISPR RNA
CuNCs	copper nanoclusters
CV	cyclic voltammetry
DBCO	dibenzocyclooctyne
DPV	differential pulse voltammetry
dsDNA	double-stranded DNA
ELISA	enzyme-linked immunosorbent assay
<i>Escherichia coli</i>	<i>E. coli</i>
E-test	epsilometer test
FRET	förster resonance energy transfer
FSY	fluorosulfate-L-tyrosine
g-FET	graphene-field effect transistor
gRNA	guide RNA
HepBV	hepatitis B virus
hpDNA	hairpin DNA
IVT	in vitro transcription
lacZa	lacZ-alpha
LAMP	loop-mediated isothermal amplification
LB	lysogeny broth
LFA	lateral flow assay
<i>Listeria monocytogenes</i>	<i>L. monocytogenes</i>

LOD	limit of detection
LSPR	localized surface plasma resonance
MB	methylene blue
MCH	6-Mercapto-1-hexanol
MOI	multiplicity of infection
PAM	protospacer adjacent motif
PCR	polymerase chain reaction
PFU	plaque-forming unit
PNA	peptide nucleic acid
QCM	quartz crystal microbalance
Qdots	quantum dots
qPCR	quantitative polymerase chain reaction
RAuNPs	raman probe-functionalized Au nanoparticles
RCA	rolling circle amplification
rNTP	ribonucleotide
RPA	recombinase polymerase amplification
RT	reverse transcription
RT-PCR	reverse-transcription PCR
RT-RPA	reverse transcription recombinase polymerase amplification
<i>Salmonella</i> Typhimurium	<i>S. Typhimurium</i>
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SCE	saturated calomel electrode
SDA	strand displacement amplification
SERS	surface-enhanced raman spectroscopy
SNP	single nucleotide polymorphism
SSBs	single-stranded DNA binding proteins
ssDNA	single-stranded DNA
<i>Staphylococcus aureus</i>	<i>S. aureus</i>
SuFEx	proximity-enabled sulfur-fluoride exchange
TCEP	tris(2-carboxyethyl)-phosphine hydrochloride
TCSV	tobacco curly shoot virus
TEM	transmission electron microscopy
tracrRNA	trans-activating crRNA
TXTL	cell-free transcription-translation

UCNPs	upconversion nanoparticles
WHO	world health organization
WT	wild type
X-gal	5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside
β -gal	β -galactosidase

Chapter 1

Introduction

Diseases such as infections and cancer are leading causes of death worldwide. According to the World Health Organization (WHO), infectious diseases are responsible for approximately 17 million deaths annually, accounting for over 30% of all deaths in low-income countries.^{20, 21} The burden of these diseases is intensified by the emergence of new pathogens, such as SARS-CoV-2,²²⁻²⁴ and the growing threat of antimicrobial resistance. In parallel, cancer remains a major global health challenge. In 2020 alone, there were 19.3 million new cancer cases and nearly 10 million cancer-related deaths worldwide, and the global cancer burden is expected to reach 28.4 million new cases by 2040.¹ Both infectious diseases and cancer require early, accurate, and accessible diagnosis to improve clinical outcomes, control disease spread, and reduce healthcare costs. Despite their distinct biological origins, they share similar diagnostic challenges, including the need to detect low levels of biomarkers in complex biological samples.² As a result, there is an increasing demand for advanced diagnostic technologies that can support early-stage disease detection across a range of clinical and field settings.

Conventional diagnostic methods have played a vital role in detecting both infectious diseases and cancer. Culture-based techniques are considered the gold standard for identifying viable bacterial pathogens, offering high specificity and the ability to assess antimicrobial resistance.⁴ However, these methods are time-consuming, often requiring days to obtain results, and they are not applicable to non-culturable organisms. Immunoassay-based methods, including enzyme-linked immunosorbent assay (ELISA)⁵ and lateral flow assays (LFA),⁷ are widely used for their simplicity and speed. Nevertheless, they often suffer from cross-reactivity, low sensitivity in early-stage disease, and inconsistent performance across pathogen strains or cancer subtypes due to variations in surface antigens or protein expression. Molecular approaches such as quantitative polymerase chain reaction (qPCR) provide higher sensitivity and specificity by amplifying target nucleic acids,⁹ but they rely on expensive instruments, skilled personnel, and controlled laboratory environments, which limit their utility in decentralized or resource-limited settings. Sequencing technologies offer comprehensive genomic information and are highly accurate but are often costly, complex, and impractical for routine or rapid diagnostics. Given these limitations, there is an

urgent need to develop novel diagnostic technologies that are sensitive, specific, rapid, cost-effective, and adaptable to a variety of testing environments.

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) systems have offered significant advances in the field of disease diagnosis.¹¹ Originally discovered as adaptive immune mechanisms in bacteria and archaea, CRISPR systems protect against invading genetic elements such as bacteriophages and plasmids. The core components of CRISPR include a Cas (CRISPR-associated) nuclease and a CRISPR RNA (crRNA), which together enable programmable, sequence-specific targeting through complementary base pairing. This precise recognition allows the Cas nuclease to cleave specific nucleic acid sequences, forming the basis of CRISPR's versatility.¹³ In diagnostics, CRISPR provides several advantages over traditional methods. It offers high specificity due to the crRNA–target base pairing, and can detect low concentrations of DNA or RNA without requiring complex instrumentation.¹⁵ Moreover, CRISPR systems are adaptable for both DNA and RNA targets, making them ideal for detecting a wide range of pathogens and genetic mutations. Among them, Class 2 CRISPR systems—such as Cas9, Cas12, Cas13, and Cas14—are particularly attractive for diagnostics due to their single-effector simplicity and ease of use.¹⁷ These features have positioned CRISPR as a powerful platform for rapid, accurate, and flexible detection of both infectious diseases and cancer-associated genetic alterations.

Building upon these advantages, this dissertation focuses on the development and application of CRISPR-integrated biosensing platforms for human health. The central objective is to design diagnostic tools that are adaptable, highly sensitive, and suitable for real-world deployment. Multiple CRISPR/Cas systems—including Cas9, Cas12, Cas13, and Cas14—are investigated for their unique capabilities in overcoming current limitations of traditional diagnostics. These platforms are applied across a range of biomedical challenges, including antimicrobial susceptibility testing, pathogen detection, and identification of cancer-related genetic mutations. The structure of this dissertation is outlined as follows:

Chapter 1 introduces the global challenges posed by diseases and the limitations of conventional diagnostic technologies. This chapter emphasizes the urgent need for rapid, sensitive, and specific detection platforms. It also introduces the CRISPR system as a versatile tool for molecular diagnostics and outlines the objectives of the thesis.

Chapter 2 reviews the current landscape of CRISPR-based biosensors. This chapter discusses the development and classification of CRISPR-based biosensing platforms, nucleic acid amplification methods, and the role of different Cas proteins (Cas9, Cas12, Cas13, and Cas14) in nucleic acid detection. Signal transduction mechanisms such as colorimetric, fluorescence, and electrochemical outputs are also discussed. This comprehensive review establishes the foundational context for the experimental chapters that follow.

Chapter 3 presents the development of a CRISPR/Cas9-mediated phage engineering platform for high-throughput antimicrobial susceptibility testing (AST). Using T4 bacteriophage as a model, the genome was edited to insert a *lacZa* reporter gene, enabling colorimetric detection of β -galactosidase expression as an indicator for bacterial viability. The resulting phage biosensor was used to assess the susceptibility of multidrug-resistant *Escherichia coli* strains in urine samples, providing a rapid alternative to traditional AST methods.

Chapter 4 describes the construction of an electrochemical CRISPR/Cas12a-based biosensor for detecting *Salmonella* Typhimurium, a major foodborne pathogen. By combining PCR amplification with hairpin DNA probes immobilized on electrodes, the assay converts CRISPR/Cas12a-mediated *trans* cleavage into a quantifiable electrochemical signal. The biosensor achieved high sensitivity in spiked poultry samples, demonstrating its potential for food safety monitoring and on-site pathogen detection.

Chapter 5 introduces a novel strategy to enhance the sensitivity of CRISPR/Cas12a-based biosensors through site-specific covalent crosslinking of the Cas12a protein and crRNA. By incorporating the unnatural amino acid fluorosulfate-L-tyrosine (FSY) into Cas12a, covalent bonding with crRNA was achieved through a sulfur-fluoride exchange reaction. The optimized Cas12a variant exhibited improved *trans*-cleavage activity, enabling sensitive detection of SARS-CoV-2 RNA in clinical samples. This approach demonstrates how protein engineering can advance CRISPR diagnostics at the molecular level.

Chapter 6 presents a dual-gene detection strategy for *E. coli* O157:H7 using a one-pot CRISPR/Cas13 biosensor. Two distinct Cas13 enzymes (LwaCas13a and PsmCas13b) were used to independently target serotype-specific genes (*rfbE*_{O157} and *fliC*_{H7}), enabling dual-channel fluorescence detection within a single reaction tube. This chapter also includes validation in a mouse infection model, confirming the biosensor's accuracy against conventional PCR. The

assay's dual-channel capability and simplified workflow support its application in clinical diagnostics.

Chapter 7 explores high-fidelity detection of single nucleotide polymorphisms (SNPs) using type V CRISPR systems (Cas12a and Cas14a), focusing on the cancer-relevant BRAF V600E mutation. A comparative study between Cas12a and Cas14a enzymes revealed superior mismatch discrimination by Cas14a. To further improve specificity, the system was integrated with blocker displacement amplification (BDA), enabling sensitive detection of mutant DNA at low variant allele frequencies in human colorectal cancer cell lines. This work demonstrates the potential of CRISPR-based SNP diagnostics in precision medicine.

Chapter 8 discusses future advancements in CRISPR-based biosensors, including Cas nuclease engineering, standardized sample pretreatment, and detection of non-nucleic acid targets. It also emphasizes amplification-free strategies, machine learning for high-throughput analysis, and portable device integration. These developments aim to improve sensitivity, specificity, and usability, expanding the impact of CRISPR diagnostics in clinical, food, and environmental applications.

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Chapter 2

Literature Review: CRISPR-based biosensors for human health

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2.1 Introduction

CRISPR (clustered regularly interspaced short palindromic repeats) technology is a promising tool to meet the needs of detecting diseases in a rapid, sensitive, and specific manner. CRISPR was discovered in bacterial and archaeal cells as an adaptive defense system against phage infections or mobile genetic elements.²⁰ In 2012, the CRISPR/Cas9 system was first demonstrated to have the ability to cleave DNA in vitro, marking a significant milestone in the field of genetic research. This breakthrough discovery quickly led to widespread application for genome editing and gene therapies.²¹⁻²⁴ In 2016, another breakthrough occurred when the CRISPR/Cas9 system was reported to have the ability to detect nucleic acids. This development opened up new possibilities for CRISPR systems to detect diseases by specifically targeting their nucleic acids. CRISPR systems can be classified into two major broad classes (class 1 and class 2) depending on the differences between Cas nucleases. Class 1 CRISPR/Cas system consists of multi-subunit nucleases complexes for the cleavage of nucleic acids. In class 2 CRISPR/Cas systems, there is only a single Cas nuclease, including type II (Cas9), type V (Cas12, Cas14), and type VI (Cas13). Due to the simplicity of the assembled CRISPR/Cas nuclease complex, class 2 CRISPR/Cas systems have been widely studied and used to detect diseases.

To highlight the recent advances of CRISPR-based biosensors for human health application, this review will cover the development of CRISPR-based biosensors to detect diseases. In addition, the selection and design of effective CRISPR systems will be highlighted, including Cas9, Cas12, Cas13, and Cas14. We will also summarize the technologies of nucleic acid amplification to detect diseases at low concentrations, and the mechanisms of signal transductions to visualize the detection results. Furthermore, the review will provide insights into future directions for developing CRISPR-based biosensors to address emerging diseases. By combining recent

advancements with future prospects, the review aims to significantly contribute to the advancement of diagnostic technologies and their impact on human health.

2.2 Development of CRISPR-based Biosensors

Advances in CRISPR technologies have made it feasible to detect infectious diseases, enabling their applications in various fields such as environmental monitoring, food safety, and clinical diagnostics. To ensure high detection sensitivity and specificity, it is important to design an effective CRISPR-based biosensor. As shown in **Figure 2-1**, the detection of infectious diseases using CRISPR-based biosensors includes target analyte identification, nucleic acid amplification, CRISPR system selection, and signal transduction. Since most infectious diseases are caused by either bacterial and/or viral pathogens, the first step is to identify the appropriate pathogens. However, complex sample matrices (such as food, blood, and feces) and trace amounts of target analytes pose challenges to the target identification process. To address this issue, the amplification is typically combined with a CRISPR-based biosensor to enhance the detection sensitivity and selectivity, including 1) DNA-to-DNA, 2) RNA-to-DNA, 3) DNA-to-RNA, and 4) RNA-to-DNA-to-RNA. The conversion between DNA and RNA can be achieved by *in vitro* transcription (IVT) and reverse transcription (RT). Theoretically, nucleic acid amplification technologies can amplify one DNA/RNA copy to one million copies within 30 min, ensuring detection sensitivity as low as one bacterial/viral pathogen.

Based on the product of nucleic acid amplification, appropriate CRISPR/Cas systems should be selected to detect either DNA or RNA. There are two important components in the CRISPR system: a Cas endonuclease and a programmed guide RNA (gRNA). The Cas endonuclease acts like scissors to cut nucleic acids, and the gRNA can navigate the Cas endonuclease to cut the target nucleic acids specifically. For Cas9 and Cas14 systems, gRNA is fused with *trans*-activating crRNA (tracrRNA) and CRISPR RNA (crRNA), in which tracrRNA can bind to nucleases and crRNA is programmable for specifically binding to a target DNA. The tracrRNA-crRNA can be integrated to be a single guide RNA, called sgRNA. On the contrary, crRNA in the Cas12 and Cas13 systems serve both functions of binding to nucleases and targeting nucleic acids. The cleavage of target DNA by Cas9 and Cas12 nucleases begins with identifying the specific protospacer adjacent motif (PAM) which is subject to the specific Cas nucleases. Cas13 nuclease can recognize and cleave RNA without the requirement of PAM. Like Cas12 nucleases, Cas14 can only cleave single-stranded DNA (ssDNA) and doesn't require PAM.

All these CRISPR systems are capable of recognizing and cleaving nucleic acids that are complementary to the crRNA, which is named *cis*-cleavage activity (specific). After the *cis*-cleavage activity, Cas12, Cas13, and Cas14 can offer additional indiscriminate ssDNA or ssRNA cleavage activity (termed as *trans*-cleavage activity, non-specific).²⁵ Due to the additional feature of *trans*-cleavage activity, Cas12, Cas13, and Cas14 offer advantages over Cas9 in detecting infectious diseases. For the CRISPR-based biosensors, the crRNA is highly target-specific, which serves as a recognition element to identify desired nucleic acids from infectious diseases. The specific recognition events can be amplified and transduced by Cas nucleases, which can be used to develop various signal transduction methods, including colorimetry, fluorescence, electrochemistry, and others. The detection performance of CRISPR-based biosensors can be evaluated by the response time, detection limit, dynamic range, and specificity.

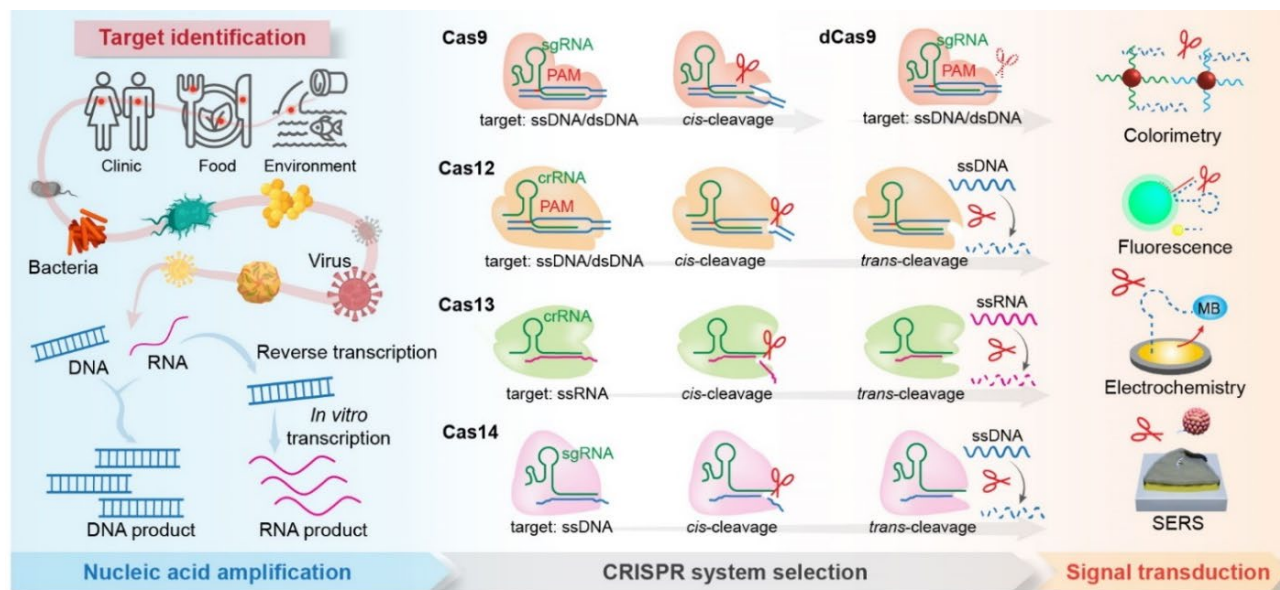


Figure 2-1 Schematic illustration of CRISPR-based biosensors.

The process of infectious disease detection using CRISPR-based biosensors involves four steps. 1) the target analyte (either bacterial and/or viral pathogens) need to be identified. 2) nucleic acid amplification techniques are employed to increase the amount of pathogen-associated nucleic acids. 3) an appropriate CRISPR system is selected to specifically recognize the amplified nucleic acid product. 4) a signal transduction strategy is utilized to transduce the specific recognition process into a measurable signal, allowing for the detection of infectious disease.

2.3 Nucleic Acid Amplification

CRISPR-based biosensors provide highly specific target recognition. However, they frequently depend on the amplification of nucleic acids to detect infectious diseases when the target analytes are present in low concentrations in the sample. Amplification is crucial to enhance the detection sensitivity of CRISPR-based biosensors, allowing them to accurately detect and identify even trace amounts of the target, such as those found in the early stages of an infection. Various amplification techniques are available and can be combined with CRISPR-based biosensors to detect infectious diseases, including polymerase chain reaction (PCR), recombinase polymerase amplification (RPA), loop-mediated isothermal amplification (LAMP), rolling circle amplification (RCA), strand displacement amplification (SDA), and many more.³¹ Each of these techniques has unique advantages and limitations, making it crucial to carefully consider the specific downstream CRISPR application when selecting an appropriate nucleic acid amplification method.

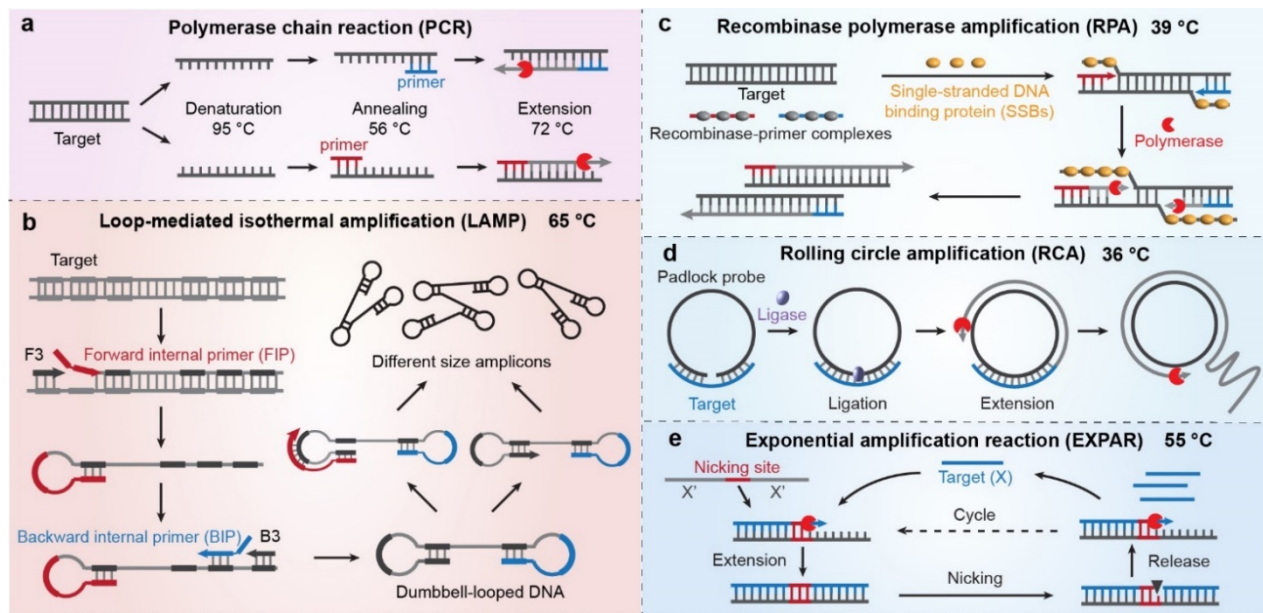


Figure 2-2 Nucleic acid amplification methods.

(a) PCR undergoes denaturalization, annealing, and extension via thermal cyclers. (b) LAMP uses at least four primers (two inner primers: FIP, BIP; two outer primers: F3, B3) to generate extensive dumbbell-structures of amplified DNA. (c) RPA utilizes recombinases and single-stranded DNA binding proteins (SSBs) for isothermal amplification. (d) RCA amplifies the target DNA using a circular DNA template. (e) EXPAR utilizes a strand displacement mechanism to exponentially amplify target sequences through repeated cycles of denaturation, annealing, extension, and nicking.

2.3.1 Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) is the gold standard and most widely used method for nucleic acid amplification. In PCR, a series of heating and cooling procedures are repeated to specifically amplify targeted double-stranded DNA (dsDNA). As shown in **Figure 2-2a**, the reaction mixture is initially heated to denature the target dsDNA, effectively separating its two strands. Subsequently, the reaction is cooled to create the optimal conditions for the primers to anneal, binding to each of the original DNA strands. After that, the temperature slightly increased to facilitate the extension of the new DNA strands from the primers. Typically, the "denaturing-annealing-extension" process is cycled around 30-35 times. In the past few years, CRISPR systems have been combined with PCR and reverse-transcription PCR (RT-PCR), generating highly sensitive and specific biosensors to detect infectious diseases.³⁴ For example, PCR-based CRISPR biosensors have demonstrated the detection of COVID-19,³⁵ H1N1 virus,³⁶ pathogenic bacteria,³⁷ antimicrobial resistance genes,³⁸ and many more analytes through the use of various CRISPR systems and readout signals. However, the requirement of thermal cycling for PCR-based amplification severely limits on-site amplification due to the reliance on expensive equipment and trained personnel. Therefore, there is a significant need to develop isothermal amplification for CRISPR-based biosensors.

2.3.2 Loop-mediated Isothermal Amplification (LAMP)

Loop-mediated isothermal amplification (LAMP) is a powerful nucleic acid amplification technique that can be used to rapidly amplify target DNA or RNA. LAMP is performed under isothermal conditions, often providing a simpler and more readily available alternative to PCR, especially for CRISPR-based sensors designed to be used on-site.³⁹ LAMP utilizes a set of four or six primers and *Bst* polymerase to form dumbbell-loop structures that facilitate subsequent cycling amplification, generating extensively large amplified products (**Figure 2-2b**). Reactions are typically carried out between 60-70°C for 30 minutes with kits commercially available from several vendors. Fluorescent signal readouts are often combined with LAMP reactions for real-time LAMP (RT-LAMP) detection of infectious diseases. In addition, LAMP has been combined with CRISPR for the specific detection of Influenza A and B virus,⁴⁰ SARS-CoV-2,⁴¹ and pathogenic bacteria.⁴² While LAMP provides several advantages to PCR, drawbacks include

complicated primer design and a high risk of false-positive signals derived from non-specific amplification and aerosol contamination.⁴³

2.3.3 Recombinase Polymerase Amplification (RPA)

Recombinase polymerase amplification (RPA) is another commercially available isothermal amplification method and can be combined with CRISPR-based biosensors to detect infectious diseases. Significantly, RPA amplification avoids thermal template denaturation, achieving exponential amplification at the low-temperature range of 37-42 °C. In this strategy, a recombinase binds to a primer to form a complex capable of recognizing and displacing the template nucleic acids (**Figure 2-2c**). A single-stranded DNA binding protein (SSB) then binds and stabilizes the displaced strand. The entire exponential amplification can be completed within 30 min. Additionally, due to the similar reaction temperatures of the RPA and CRISPR nucleases, amplification and CRISPR-based detection can be performed simultaneously, drastically simplifying potential detection mechanisms.⁴⁴ RPA-based CRISPR systems have successfully detected SARS-CoV-2⁴⁵ and various pathogenic bacteria, including *Staphylococcus aureus*,⁴⁶ *Trichomonas vaginalis*,⁴⁷ *Salmonella* Typhimurium,^{48, 49} and *Brucella* spp.. Limitations of RPA amplification include the lack of specialized primer design software, limited availability of commercial kits, and the use of additional reagents, including recombinase enzymes and SSBs, which increase the cost and complexity of the reaction.

2.3.4 Rolling Circle Amplification (RCA)

Some other amplification methods provide unique versatility in certain situations. Among them, rolling circle amplification (RCA) has demonstrated highly sensitive and specific amplification of circular DNA. As shown in **Figure 2-2d**, the basic procedure of RCA is presented, including ligase binding, primer extension, and strand displacement amplification. The target nucleic acid pairs with a linear probe (padlock) through ligation, forming a circular template. Subsequently, polymerase extends the original target sequence as it moves around the template, creating a long linear nucleic acid strand complementary to the template nucleic acid.⁵⁰ Uniquely, this process can produce long single-stranded DNA/RNA molecules, making it highly compatible with the integration of Cas14 systems.⁵¹ Despite the successful developments of RCA-based CRISPR biosensors for detecting several infectious diseases,⁵² it still encounters challenges in pricy padlock probes and non-specific amplification.

2.3.5 Others

Other isothermal amplification methods have been combined with CRISPR systems to detect infectious diseases, including exponential amplification reaction (EXPAR),⁵⁴ strand displacement amplification (SDA),⁵⁰ nicking-enzyme amplification (NEA),⁵⁵ recombinase-aided amplification (RAA),⁵⁶ and hybridization chain reaction (HCR).⁵⁷ For example, EXPAR employs a combination of nicking and strand displacement for extremely sensitive isothermal amplification of target DNA. As shown in **Figure 2-2e**, the EXPAR template is designed to have two repeat regions (X') that perfectly match the target sequence (X). These repeat regions are separated by a cleavage site recognized by a nicking enzyme, which enables the template to be cleaved during the amplification process. EXPAR is initiated when target DNA pairs with the template sequence, resulting in the formation of a double-stranded duplex. Subsequently, DNA polymerase extends the duplex, generating an extended double-stranded DNA with a nicking site. In the presence of a nicking enzyme, the target strand is cleaved and then released through strand displacement mediated by DNA polymerase. After that, the displaced target strand is complementary to another template, initiating another round of amplification. EXPAR is typically carried out between 55-65 °C, even if the nicking enzyme works well at lower temperatures.¹⁸ EXPAR-CRISPR sensors have been utilized with a variety of Cas enzymes for the detection of DNA mutations,⁴ creatine kinase MB,¹¹ and microRNAs.³ While EXPAR is extremely rapid and robust, the method requires an extremely complicated primer design and often results in non-specific interactions, leading to a high background amplification. Therefore, EXPAR is not a widely used amplification technique.

2.4 CRISPR/Cas Systems for Sensing Nucleic Acids

Benefiting from high programmability and specificity, CRISPR/Cas systems have become a powerful tool for infectious disease detection. In combination with nucleic acid amplification technologies mentioned above, CRISPR/Cas systems are designed to achieve a highly sensitive and specific detection of disease-related nucleic acids. However, the intrinsic differences of the CRISPR Cas nucleases vary in the detection methods, particularly for target types and *trans*-cleavage activities. Even when using the same CRISPR Cas nuclease, the underlying strategies of these detections may be entirely different. The following sub-sections will introduce various CRISPR/Cas systems and their underlying detection strategies for sensing disease-related nucleic acids.

2.4.1 CRISPR/Cas9 System

CRISPR/Cas9 is the first CRISPR system to be used for infectious disease detection.⁹ The core of its detection mechanism is the *cis*-cleavage activity of the Cas9 nuclease to cleave the target DNA, which is closely related to its two structural domains, namely HNH and RuvC. After recognizing the PAM from the target DNA, the Cas9 nuclease binds the target with HNH and RuvC domains and triggers the cleavage activity on the target sequence. Besides, the endonuclease-deactivated Cas9 (dCas9) is a mutant form of Cas9 nuclease. Through silencing two mutations in the HNH and RuvC domain, the cleavage activity of Cas9 is removed while maintaining the binding activity toward the target DNA. In recent years, several CRISPR-based biosensors have been developed using Cas9 and dCas9.

Identification of single nucleotide mismatch. Since the CRISPR/Cas9 system can recognize and perform blunt end cuts on target dsDNA, the CRISPR/Cas9-based biosensors usually use

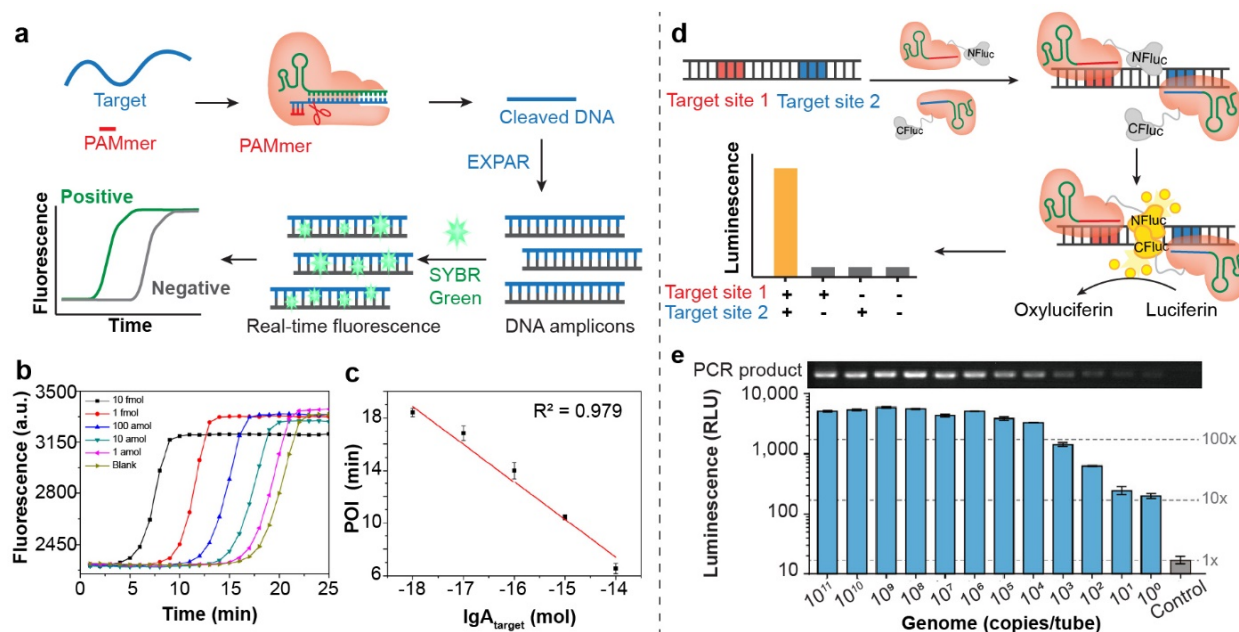


Figure 2-3 CRISPR/Cas9-based sensing strategies.

(a) Principle of CRISPR-Cas9 mediated isothermal amplification method for the identification of single nucleotide mismatch (PAMmer: PAM-presenting oligonucleotide). (b) Real-time fluorescence intensity curve responding to different DNA concentrations. (c) Calibration curve of point of inflection (POI) toward DNA concentrations. (Reproduced with permission from ref. ^{3, 4}, Copyright 2018, American Chemical Society) (d) Design of CRISPR/dCas9-based biosensor for the detection of *Mycobacterium tuberculosis* (e) Detection sensitivity of CRISPR/dCas9 system targeting the *Mycobacterium tuberculosis* 16S rRNA gene. (Reproduced with permission from ref. ^{12, 13}, Copyright 2017, American Chemical Society)

cleaved DNA as the template for further amplification. As shown in **Figure 2-3a**,⁵ in the presence of PAM-presenting oligonucleotide (PAMmer), the target ssDNA was recognized and cleaved by the CRISPR/Cas9 system, releasing the cleaved DNA for cyclic amplification with the assistance of nicking endonuclease. As a result, numerous DNA amplicons were generated, and the signal was monitored by real-time fluorescence (**Figure 2-3b**). This method achieved a detection limit of 0.82 amol within 1 hour (**Figure 2-3c**), providing superior specificity in the identification of single-base nucleotide mismatch. Furthermore, this method has been successfully extended to the detection of DNA methylation as well as *L. monocytogenes* total RNA, demonstrating its versatility in various application scenarios.

Deactivated Cas9 (dCas9). Due to the removal of its endonuclease activity, dCas9 can recognize and bind to target nucleic acids without causing any cleavage. The dCas9-based biosensors rely on synthetic biology to bring split enzymes at the terminal of dCas9 together. For example, a recent study investigated a paired dCas9 reporter system to re-functionalize split firefly luciferase for the detection of infectious diseases (**Figure 2-3d**).¹⁰ The luciferase was divided into two parts and separately linked with dCas9. These recombinant dCas9 nucleases were programmed by sgRNA complementary to different segments of target DNA at a short distance. In the presence of the target nucleic acid, two recombinant dCas9-sgRNA complexes recognized and bound the target DNA sequence, integrating two parts of luciferase and triggering the bioluminescent reaction. This detection strategy allowed such assays with paired dCas9 to detect *Mycobacterium tuberculosis* with a detection limit of one genomic copy (**Figure 2-3e**).

2.4.2 CRISPR/Cas12 System

Although several studies have utilized CRISPR/Cas9 and CRISPR/dCas9 for the detection of infectious diseases, the complex design and tedious procedure hinder the transduction of the CRISPR/Cas9 system alone from target identification to readable outputs. Taking the gift of *trans*-cleavage toward nonspecific oligonucleotides by the RuvC domain, the Cas12 nucleases are able to provide direct signal outputs upon target identification, which greatly simplifies the detection procedure and extends the potential applications for infectious disease diagnosis.

Photoactivable CRISPR/Cas12 system. In the conventional CRISPR/Cas12 system, Cas12 nucleases are triggered immediately in the presence of target DNA. To detect target DNA at low concentrations, nucleic acid amplifications are combined with the CRISPR/Cas12 system. But the conventional one-pot method suffered from poor compatibility because the activated

CRISPR/Cas12 system could prevent nucleic acid amplification. To overcome this problem, a photoactivatable CRISPR/Cas12-based biosensor was developed for the detection of SARS-CoV-2 (**Figure 2-4a**).⁵ The SARS-CoV-2 RNA was amplified by RT-RPA, which wasn't affected by the CRISPR/Cas12 system because the crRNA was temporarily blocked by a protective oligo. Once the RPA reaction was completed, the protective oligo was broken down by ultraviolet light at 365 nm, followed by activating the CRISPR/Cas12 reaction and generating fluorescence signals. Compared with the conventional one-pot CRISPR assay, this method achieved more than two orders of magnitude higher sensitivity with a limit of detection of 10 copies (**Figure 2-4b-c**).

Split Cas12 nuclease. With the development of intelligent detection, the split Cas nuclease was explored to construct the molecular logic gate, providing an advanced approach for the precise control of biological reactions. As shown in **Figure 2-4d**, a two-input Boolean AND logic gate was developed through expressing a pair of split Cas12 nuclease with leucine zipper domains.¹² Without disrupting the native structure, the Cas12 protein was artificially split into two parts (N-terminal and C-terminal Cas12) as two inputs in the AND logic gate. A pair of leucine zippers were fused to the split nuclease for inducing the spontaneous assembly of the two parts. The operation

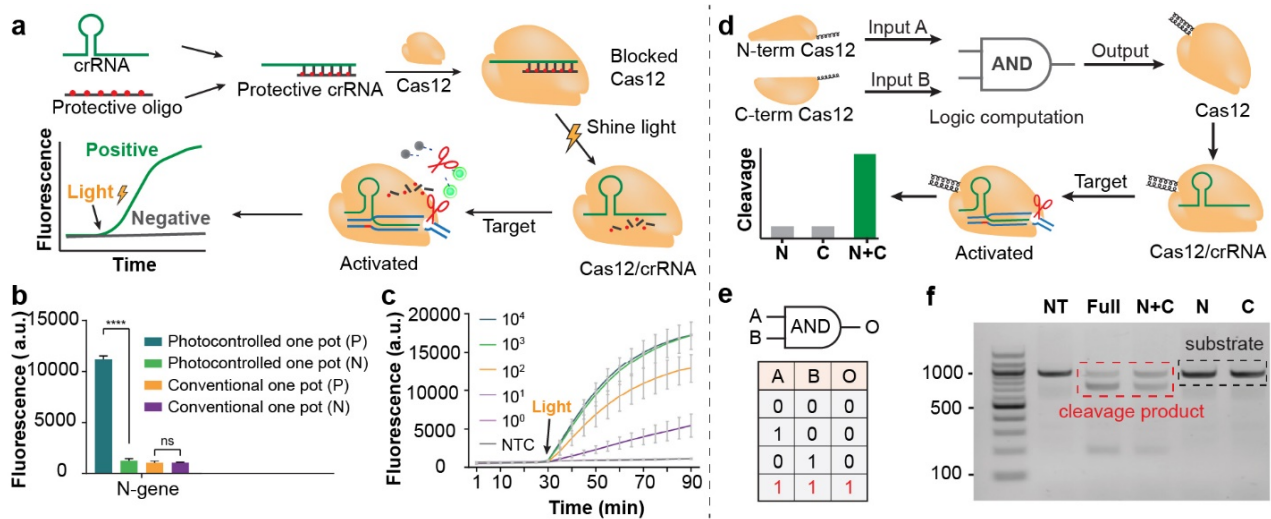


Figure 2-4 CRISPR/Cas12-based sensing strategies.

Schematic illustration of photoactivatable CRISPR/Cas12 system to detect SARS-CoV-2. (b) Comparison of the photoactivatable one-pot system with the conventional one-pot system. (c) Fluorescence signal response of photoactivatable system toward different copies of SARS-CoV-2 N gene. (Reproduced with permission from ref. ¹, Copyright 2022, National Academy of Sciences, USA) (d) Principle of the AND logic gate to detect nucleic acids using split Cas12 enzyme with leucine zipper. (e) The algorithm of 2-input AND gate. (f) Gel electrophoresis results of split Cas12-mediated cleavage of dsDNA (960 bp). (Reproduced with permission from ref. ^{10, 11}, Copyright 2020, Elsevier)

mechanism of the molecular AND logic gate follows a binary operation (**Figure 2-4e**), where the presence and absence of inputs are defined as 1 and 0, respectively. For the output, the mature Cas12 enzyme with cleavage activity was indicated as 1, while 0 represents the incomplete Cas12 enzyme without cleavage activity. Only in the presence of both inputs, the Cas nuclease can be reassembled and interacted with crRNA, serving as a functional nuclease to cleave the target DNA. This split Cas12-based logic gate system was used to cleave the genomic CSCR4 locus of HEK293T cells (**Figure 2-4f**). Compared with the full-length Cas12 nuclease, the reassembled Cas12 nuclease (N+C) showed the comparable cleavage ability of target DNA fragments, offering higher flexibility and programmability.

2.4.3 CRISPR/Cas13 System

Cas13 is a type VI nuclease and is starkly different from the previously introduced nucleases because of two HEPN domains that act as cleavage sites to target RNA without the requirement of PAM. Besides, some of the Cas13 enzymes have specific dinucleotide cleavage motif preferences

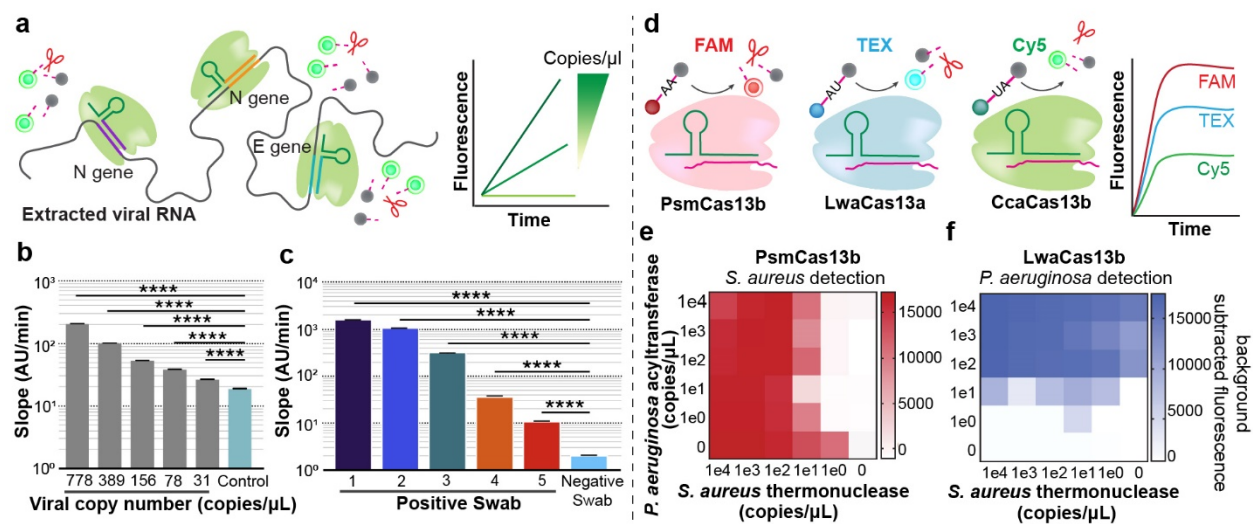


Figure 2-5 CRISPR/Cas13-based detection strategies.

(a) Schematic of tandem CRISPR/Cas13 system to detect SARS-CoV-2. (b) The detection sensitivity of tandem CRISPR system to different concentrations of SARS-CoV-2 RNA. (c) The detection performance of tandem CRISPR system on patient nasopharyngeal swabs. (Reproduced with permission from ref. , Copyright 2021, Elsevier) (d) Design of CRISPR/Cas13-based multiplexed detection of pathogenic bacteria using dinucleotide preferences of Cas13 nucleases. (e-f) Multiplexed detection of *S. aureus* thermonuclease and *P. aeruginosa* acyltransferase with LwaCas13a and PsmCas13b, respectively. (Reproduced with permission from ref. , Copyright 2018, American Association for the Advancement of Science)

that facilitate the design of multiplexed detection. Due to its unique properties, Cas13 has been widely applied in infectious disease diagnosis.

Tandem CRISPR system. To achieve high sensitivity without pre-amplification, a tandem CRISPR/Cas13-based biosensor was developed for the rapid detection of SARS-CoV-2 (**Figure 2-5a**).²⁶ By integrating three crRNAs targeting the N gene or E gene of SARS-CoV-2 genomic RNA, the Cas13 nucleases were synergistically activated, resulting in the cleavage of more fluorescent reporters and the improvement of detection sensitivity. Without any nucleic acid amplification, the tandem CRISPR system reached a detection limit of 31 copies/ μ L (**Figure 2-5b**) and correctly identified the nasal swab samples from positive patients (**Figure 2-5c**). Moreover, the utilization of multiple crRNAs toward different regions of the virus can safeguard against the potential false-negative results due to viral mutations.

Multiplexed detection. In contrast to the indiscriminate *trans*-cleavage activity of Cas12, the Cas13 nucleases have unique dinucleotide preferences for *trans*-cleavage. Recent studies found that the *trans*-cleavage activities of LwaCas13a, CcaCas13b, and PsmCas13b could all be independently monitored with dinucleotide reporters of AA, AU, and UA, respectively.² As shown in **Figure 2-5d**, a triplex detection method was developed using three different fluorophores. Taking advantage of these dinucleotide preferences of three Cas13 nucleases, a multiplexed detection assay with two fluorescent channels was explored for the simultaneous detection of *Pseudomonas aeruginosa* acyltransferase gene and the *Staphylococcus aureus* thermonuclease gene (**Figure 2-5e-f**). Impressively, the detection limit of this assay reached as low as 2 aM, demonstrating the high sensitivity of Cas13-based multiplexed detection assay and its capability for precise and reliable detection of infectious disease.

2.4.4 CRISPR/Cas14 System

As a compact type V nuclease, Cas14 shares a similar RuvC domain with Cas12 nuclease. The main feature of Cas14 is that it recognizes ssDNA without PAM, which serves as a double-edged sword in the development of the detection strategies for infectious diseases. Since it is independent of the PAM sequence, the design of target sequences is much more flexible than other Cas nucleases, allowing the detection of single-nucleotide polymorphisms (SNP) to be more feasible. However, the genetic code of most organisms in nature is dsDNA or RNA, which limits the applications of the CRISPR/Cas14-based detection systems.

PAM independent detection. The PAM-independent characteristic provides the Cas14 enzyme with the ability for highly reliable SNP recognition. As shown in **Figure 2-6a**, a Cas14-mediated ssDNA targeting CRISPR system (Cas14-DETECTR) was designed to detect the human HERC2 gene, which contained a single-nucleotide difference between brown-eyed and blue-eyed individuals.^{58, 59} To generate the ssDNA for the Cas14 nuclease, the target gene was amplified by a phosphorothioate (PT)-modified primer, and then the T7 exonuclease was used to degrade the non-PT modified DNA strand (**Figure 2-6a-b**). When designing the crRNA toward the blue-eyed sequence, the Cas14-DETECTR showed superior SNP recognition ability with almost no background signal interference, while the Cas12-DETECTR failed to distinguish between two ssDNA sequences (**Figure 2-6c**). These results demonstrated that the Cas14-DETECTR expanded the toolbox for PAM-independent detection of SNP.

Aptamer-assisted CRISPR detection. Due to their high flexibility and specificity, aptamers offer alternative options for the design of CRISPR/Cas14-based biosensors. As shown in **Figure**

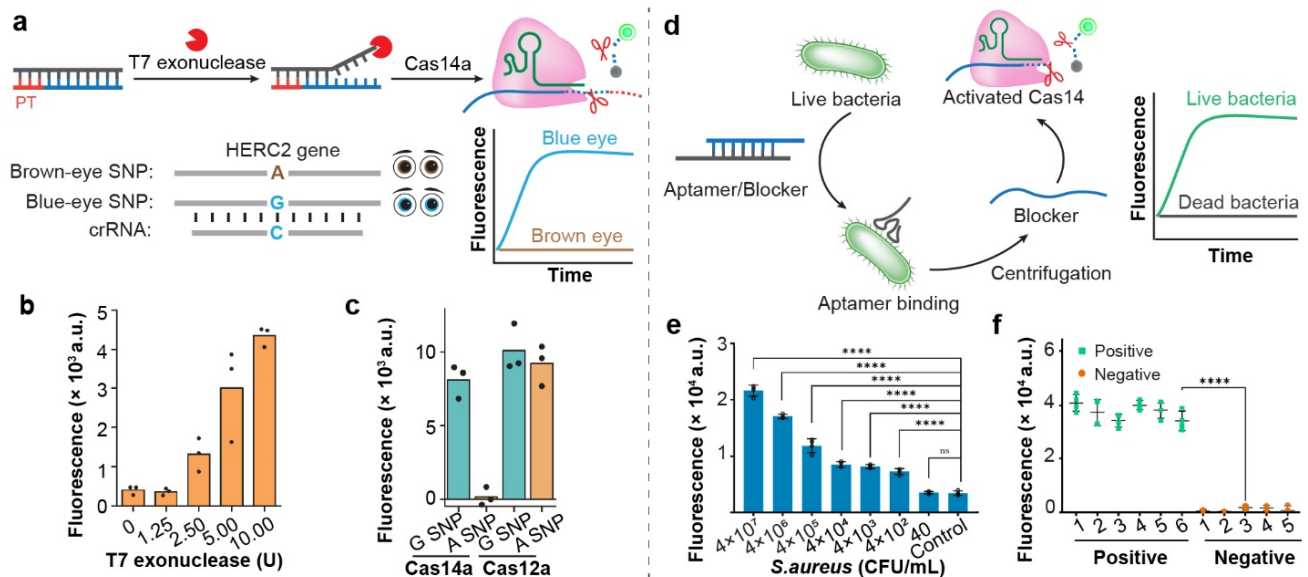


Figure 2-6 CRISPR/Cas14-based detection strategies.

Principle of CRISPR/Cas14-DETECTR for the detection of Single nucleotide polymorphisms (SNP). (b) The effect of different concentration T7 exonuclease in CRISPR/Cas14-DETECTR. (c) Comparison of the sensitivity of Cas14a-DETECTR and Cas12a-DETECTR for the detection of SNP in HERC2 gene. (Reproduced with permission from ref. ⁵, Copyright 2018, American Association for the Advancement of Science) (d) Design of aptamer-assisted CRISPR/Cas14 system for the detection of bacteria. (e) The detection sensitivity of *S. aureus* using the aptamer-assisted CRISPR/Cas14 system. (f) *S. aureus*. detection in clinical samples using the aptamer-assisted CRISPR/Cas14 system (Reproduced with permission from ref. , Copyright 2022, Elsevier)

2-6d, aptamer-assisted Cas14 biosensors can be applied to detect live *Staphylococcus aureus* (*S. aureus*). The aptamer and blocker hybridized to form a duplex strand DNA. In the presence of *S. aureus*, the aptamer binds specifically to the target bacterial cell, releasing the blocker to trigger the *trans*-cleavage activity of the CRISPR/Cas14a system. With the assistance of the aptamer and blocker, this strategy simplified the detection process without the need of generating bacterial ssDNA through a series of tedious amplification and enzymatic digestion. By correlating the fluorescence intensity with bacterial concentration, it can distinguish live *S. aureus* with a detection limit of 400 CFU/mL (**Figure 2-6e**), and 100% accuracy in the clinic samples (**Figure 2-6f**).

2.5 Signal Transduction Mechanisms

Signal transduction is an important step to enable the output of sufficient signals for the detection of infectious diseases. So far, various signal transducers have been developed for CRISPR-based biosensors, including colorimetry, fluorescence, and electrochemistry. The following section discusses the most popular signal transduction mechanisms, each categorized based on their unique mechanisms.

2.5.1 Colorimetry

Due to their simplicity and visual readability, colorimetric biosensors have received wide attention for the point-of-care testing of infectious diseases. As optical sensors, colorimetric biosensors mainly rely on the color change caused by the target analytes. In recent years, the integration of colorimetric biosensors with smartphones has facilitated the quantification of color changes in the field. Taking the outstanding advantages of colorimetric assays, CRISPR-based colorimetric biosensors have been developed to detect infectious diseases.

Gold Nanoparticles (AuNPs). Due to the localized surface plasma resonance (LSPR) effect, AuNPs have been widely used as ideal probes for colorimetric biosensors. By changing the distances between AuNPs, the solution can display a range of colors from red to blue.⁸ Based on this distinct optical property, AuNPs have been applied as the signal transducer for CRISPR-based colorimetric biosensors. As shown in **Figure 2-7a**, ssDNA-modified AuNPs remained stabilized via the strong electrostatic interactions between AuNPs.¹ The addition of ssDNA crosslinkers can shorten the distance between AuNPs, changing the solution color from red to purple. In the presence of the *Salmonella*-associated target gene (*invA*), the CRISPR/Cas12 system was activated to degrade ssDNA crosslinkers into short oligo pieces. The degraded ssDNA crosslinkers failed to

aggregate well-dispersed AuNPs, remaining the red color for the solution. The dispersed and crosslinker-induced aggregated AuNPs are characterized using transmission electron microscopy (TEM) (**Figure 2-7b**). Using this strategy, the detection limit of *Salmonella* was as low as 10 CFU/mL, which can be observed using naked eyes and measured using a spectrophotometer (**Figure 2-7c-d**). Similar CRISPR-based colorimetric biosensors have been applied to detect various infectious diseases.⁶⁰ In addition to AuNPs, other plasmonic nanomaterials (e.g., silver nanoparticles, silver nanoprisms, gold nanorods, and gold nanostars) could also be combined with various CRISPR systems to detect infectious diseases.

Lateral Flow Assay (LFA). Over the past two decades, LFA has become one of the most widely used devices for the detection of infectious diseases. Instead of using antibodies as recognition elements, DNA hybridization combined with CRISPR systems has been developed in various infectious disease detection assays. As shown in **Figure 2-7e**, the control and test lines were modified with streptavidin and anti-rabbit antibodies, respectively. In the absence of target

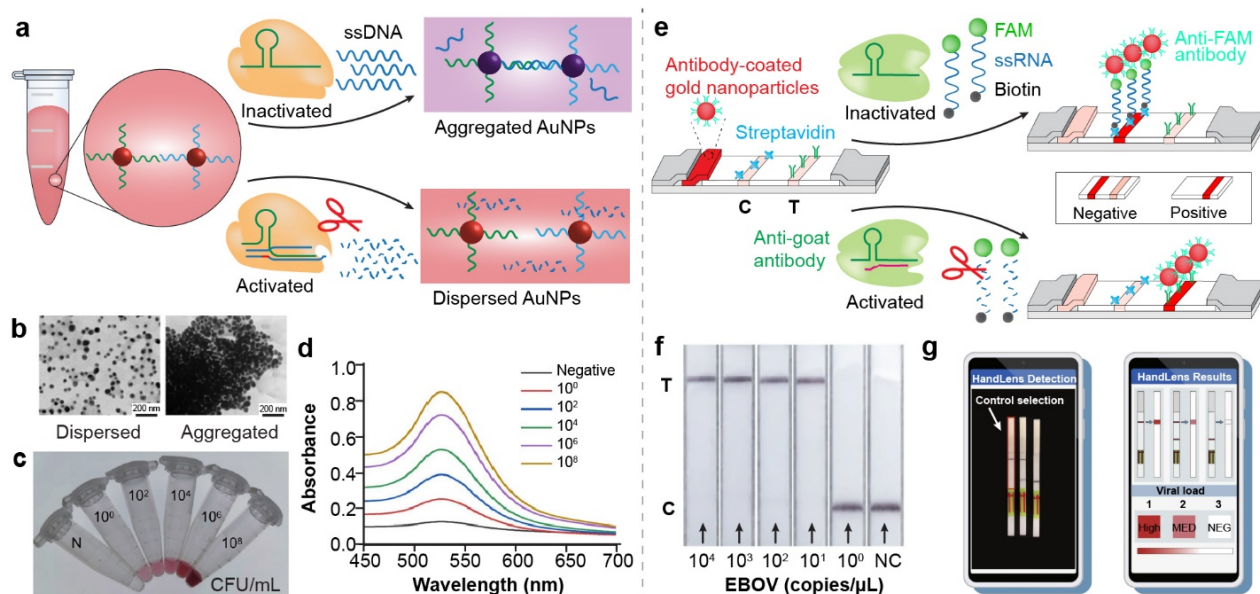


Figure 2-7 CRISPR-based colorimetric biosensors to detect infectious diseases.

(a) Principle of AuNPs-based colorimetric assay to detect *Salmonella*. (b) TEM images of dispersed and aggregated AuNPs. (c-d) Photographs and UV-vis spectra of AuNPs to detect different concentrations of *Salmonella*. , Copyright 2021, American Chemical Society) (e) Schematic illustration of LFA-based detection of SARS-CoV-2. (f). Detection limit of a serial dilution of Ebola virus (EBOV) synthetic DNA, in which C and T indicate control and test line, respectively. (g) Strip reader APP to analyze diagnostic results. The raw image data are sent to a backend server that runs the signal detection algorithm and returns the binary and semi-quantitative predictions of each strip. (Reproduced with permission from ref. , Copyright 2020, Springer Nature)

Ebola virus (EBOV) analytes, the undamaged biotin-ssRNA-FAM probes could bind the AuNPs conjugated with anti-FAM antibody- to streptavidin on the control line, resulting in a red band. Once the biotin-ssRNA-FAM probes were cleaved by the activated CRISPR/Cas13 system, the anti-FAM antibody-conjugated AuNPs were captured by the anti-rabbit antibodies on the test line, indicating the presence of target analytes. Combined with RT-RPA and transcription of EBOV RNA, this assay allowed to detection of EBOV as low as 10 viral copies per uL (**Figure 2-7f**).⁶ In addition, a smartphone app (HandLens) has been developed to interpret the colorimetric results and provided semi-quantification of the target viral concentration. As shown in **Figure 2-7g**, the smartphone APP can capture and analyze an image of one or more lateral flow strips for the semi-quantification of viral concentrations. This assay detected real samples with 93% accuracy, 91% sensitivity, and 100% specificity from a total of 21 strips.

2.5.2 Fluorescence

Due to their high sensitivity, fluorescent biosensors have been widely developed for the detection of various analytes. The concepts of most fluorescent biosensors are based on either fluorescence quenching or recovery/enhancement that is induced by the target analytes. To date, many fluorescent biosensors have been introduced to CRISPR systems for the detection of infectious diseases.

Fluorophore. The typical fluorescence probes for CRISPR-based biosensors are short ssDNA or ssRNA labeled with fluorophores and quenchers on each end, named ssDNA/RNA-FQ probes. Due to the short sequence of the ssDNA/ssRNA, the fluorophores are close to the quenchers, resulting in fluorophore quenching. After the *trans*-cleavage activity from CRISPR system toward ssDNA/RNA-FQ probes, fluorophores and quenchers are separated, resulting in fluorescence recovery. As shown in **Figure 2-8a**, dsDNA reporters have been developed to expand the reporter toolbox for CRISPR/Cas12-based biosensors.⁶¹ In this design, the fluorophore and quenchers were modified at opposite ends of each strand, respectively. In the presence of an activated Cas12 nuclease, the dsDNA reporter was degraded by the *trans*-cleavage activity, leading to an increase in fluorescence signal. Compared to the traditional linear ssDNA reporters, the dsDNA reporters showed a more stable fluorescence background that did not fluctuate significantly over time (**Figure 2-8b**). Under the optimized condition, the Cas12-based biosensor with dsDNA reporter was used to detect tobacco curly shoot virus (TCSV) DNA and hepatitis B virus (HepBV) DNA. This CIRSPR-based biosensing method has a significant signal response toward 50 pM targets,

proving that dsDNA can broaden the fluorescence reporter library for CRISPR/Cas systems (Figure 2-8c).

Fluorescent Nanomaterial. Given the advantages of easy synthesis, photostability, and low fluorescent background, fluorescent nanomaterials have been used as ideal probes for fluorescence biosensors, including copper nanoclusters (CuNCs), upconversion nanoparticles (UCNPs), quantum dots (QDots), and carbon dots (CDs).¹⁵ Among these nanomaterials, QDots offer excellent optical and electronic performances, including stable fluorescence intensity, high quantum yields, and a narrow emission spectrum. As shown in Figure 2-8d, a QDot-nucleic acid hairpin (QD-HP) probe was developed to detect nucleic acids. A peptide-peptide nucleic acid (peptide-PNA) was designed to hybrid with fluorescence-labeled DNA or RNA hairpins, and the resulting unit can self-assemble on the surface of ZnS-coated QDots. Based on the Förster

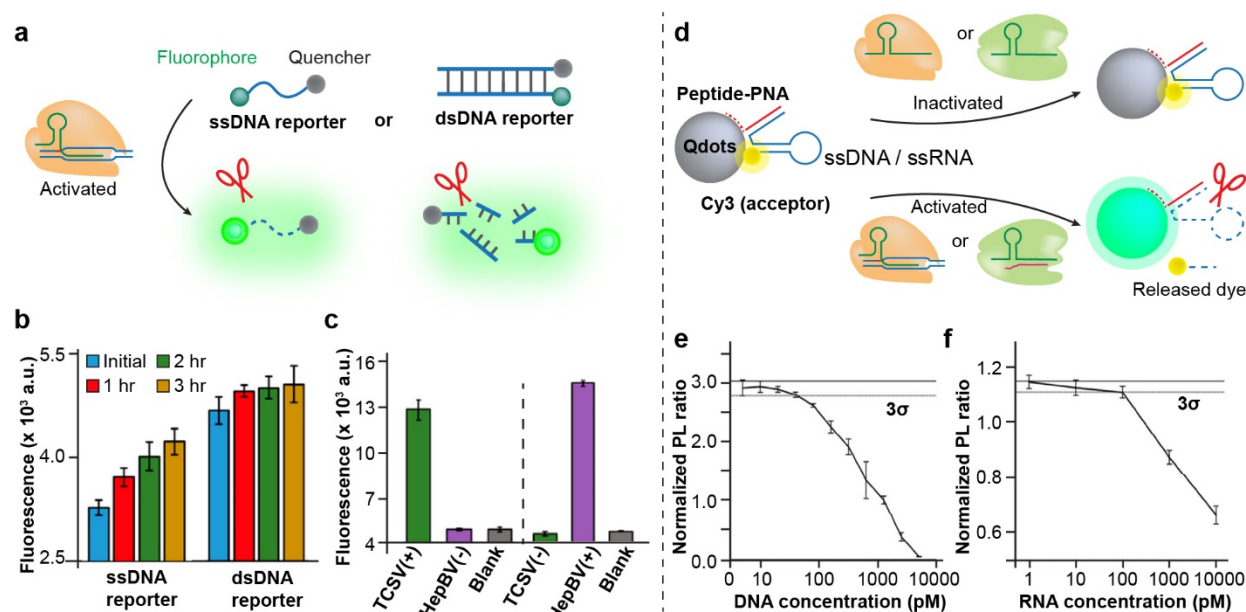


Figure 2-8 CRISPR-based fluorescent biosensors to detect infectious diseases.

(a) Design of ssDNA and dsDNA reporters for CRISPR *trans*-cleavage activity. (b) Comparison of fluorescence background of ssDNA and dsDNA reporter probes. (c) CRISPR-based fluorescence detection of tobacco curly shoot virus (TCSV) and hepatitis B virus (HepBV). (Reproduced with permission from ref. ^{1,2}, Copyright 2022, American Chemical Society) (d) Schematic illustration of QD-HP reporter for the detection of nucleic acids (DNA or RNA). Prior to the *trans*-cleavage of CRISPR systems, the Qdot is quenched by the surrounding Cy2 dye through FRET. Upon cleavage, the dyes are released and the Qdot fluorescence is recovered. (e-f) Plots of the normalized PL ratio toward the target DNA and RNA concentrations, respectively. (Reproduced from ref. ^{14, 15}, Copyright 2020, American Chemical Society)

resonance energy transfer (FRET), QDots served as an ideal donor to a fluorescence dye (Cy3). In the presence of an activated CRISPR system, the hairpin nucleic acids can be cleaved and released, enabling ratiometric detection of target DNA or RNA. In this strategy, the developed probes were used to detect DNA and RNA with a detection limit of 40 pM and 100 pM, respectively (Figure 2-8e-f).

2.5.3 Electrochemistry

Because of their easy operation, rapid signal output, and sensitive response, electrochemical biosensors are another powerful tool to detect infectious diseases. In most cases, the change of electrical signals (potential or current) is due to the changes in electron transfer on the electrode surface, which can be correlated to the analyte concentration. To date, several CRISPR systems

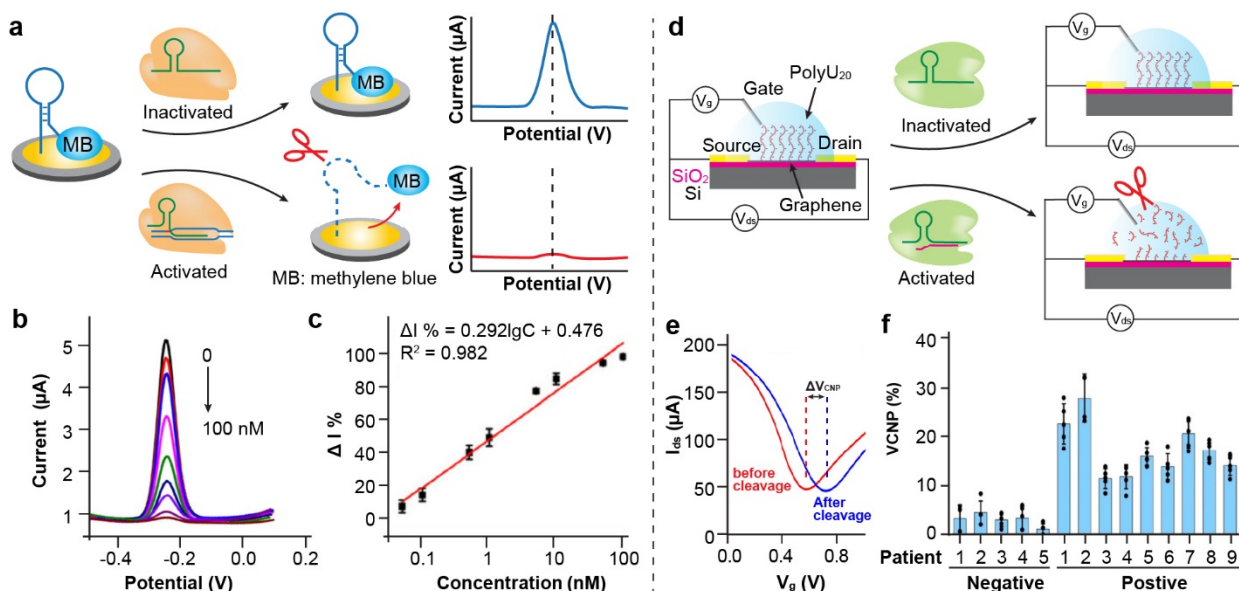


Figure 2-9 CRISPR-based electrochemical biosensors to detect infectious diseases.

(a) Schematic illustration of CRISPR-based electrochemical biosensors to detect nucleic acids (MB: methylene blue). (b) Typical differential pulse voltammetry (DPV) curve responding to different DNA concentrations. (c) The calibrated ΔI % towards target DNA concentration. (Reproduced with permission from ref. ^{6, 7}, Copyright 2020, American Chemical Society) (d) Schematic illustration of graphene-field effect transistor (g-FET) to detect nucleic acids. (e-f) Representative of g-FET before and after the *trans*-cleavage of CRISPR system. The source-drain current (I_{ds}) were monitored while keeping a constant source-drain voltage ($V_{ds} = 100$ mV). (Reproduced with permission from ref. ^{16, 17}, Copyright 2022, Wiley-VCH GmbH)

(including Cas9, Cas12, and Cas13) have been used to develop electrochemical biosensors for the detection of infectious diseases.

Electrochemistry. In CRISPR-based electrochemical biosensors, the electrode surfaces are usually modified with oligonucleotides labeled with electrochemical tags, such as methylene blue (MB). As shown in **Figure 2-9a**, the electrodes were modified with MB-labeled hairpin DNA (MB-hpDNA).⁷ Due to the specific design, the electrochemical active compounds (MB) were close to the electrodes, shortening the electron tunneling distance between MB and the electrode surface. With activated CRISPR/Cas12, the hpDNA was degraded and the MBs were detached from the electrode surfaces, resulting in a decrease of electrical response. Under the optimal conditions, the electrochemical biosensor was employed to detect human papillomavirus (HPV)-related DNA. As shown in **Figure 2-9b**, the differential pulse voltammetry (DPV) curves showed a gradual decrease with increased concentration of the target DNA. This strategy allowed for the detection of unamplified target DNA at concentrations as low as 30 pM within 60 min, and the dynamic range was from 50 pM to 100 nM (**Figure 2-9c**).

Field Effect Transistor (FET). FET has emerged as a promising technology for the detection of infectious diseases. A typical FET contains three electrodes (including gate, source, and drain electrodes), in which the gate electrode provides an electric field to control the current flow from the source electrode to the drain electrode.⁷ Graphene as an exceptional semiconductor material has been widely used in FET. As shown in **Figure 2-9d**, an ultrasensitive g-FET for nucleic acids detection has been reported using CRISPR/Cas13 system.¹⁴ Negatively charged reporters (polyU₂₀) were modified on the surface of graphene. In the presence of the activated CRISPR/Cas13 system, the polyU₂₀ probes were cleaved into small pieces, decreasing the number of electrons carried in the graphene channel. Consequently, a right shift of V_{CNP} was observed, which was correlated with the target RNA concentrations (**Figure 2-9e**). In this detection strategy, clinic samples were detected, including 5 negative and 9 positive patient samples. Using a threshold of two standard deviations above the mean obtained from negative clinic samples, the developed CRISPR/Cas13 g-FET was able to distinguish between all 5 negative and 9 positive samples with 100% accuracy.

2.5.4 Other Signal Transduction Mechanisms

In addition to the signal transduction mechanisms described above, other novel strategies have been combined with CRISPR systems to detect infectious diseases, including chemiluminescence, surface-enhanced Raman spectroscopy (SERS), nanopore, etc.

Surface-enhanced Raman Spectroscopy (SERS). SERS is a Raman signal based on the enhanced localized surface plasmon resonances of noble metals (e.g., silver and gold).¹⁻³ Because of its extremely high sensitivity and rich molecular fingerprints, SERS has been used to detect a single molecule. Similarly, the combination of SERS and CRISPR/Cas systems is a promising strategy to sensitively detect infectious diseases. As shown in **Figure 2-10a**, the SERS substrate was coated with graphene oxide and triangle gold (Au) nanoflowers (GO-TANF), which can offer extremely strong electromagnetic and chemical enhancement. The ssDNA-immobilized Raman

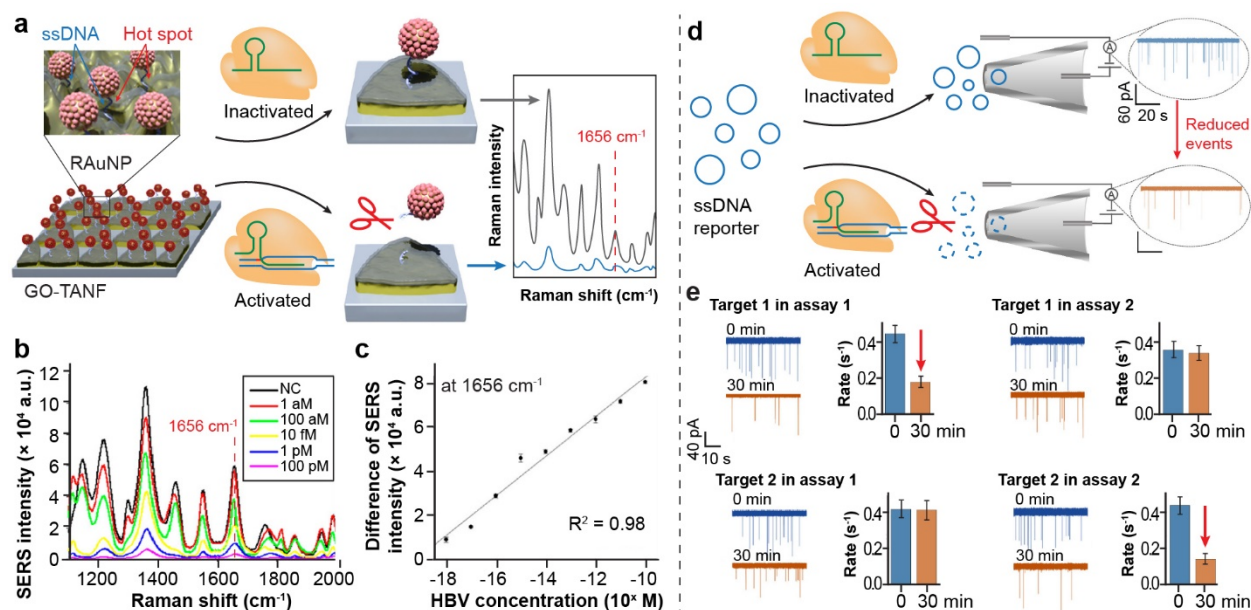


Figure 2-10 Other CRISPR-based novel biosensors to detect infectious diseases.

(a) Schematic illustration of the SERS-based strategy to detect viral DNA. RANP: Raman probe-modified AuNP. GO-TANF: graphene oxide-triangle Au nanoflower. (b) Raman spectra of the RANP-modified GO-TANF array with varying concentrations of hepatitis B virus (HBV) DNA. (c) the linear relationship of the decrease of SERS intensities towards the concentrations of HBV DNA. (Reproduced with permission from ref. ^{8,9}, Copyright 2021, American Chemical Society) (d) Schematic illustration of solid nanopore sensors to detect HIV. With the *trans*-cleavage of circular ssDNA reporter, fewer events are recorded. (e) Detection specificity of HIV using CRISPR-based nanopore sensors. Only specific combinations (Assay 1 - Target 1 and Assay 2 - Target 2) lead to the degradation of circular ssDNA reporters. (Reproduced with permission from ref. , Copyright 2020, American Chemical Society)

probe-functionalized Au nanoparticles (RAuNPs) on the GO-TANF can provide strong SERS intensity. As a proof-of-concept, the developed RAuNP-modified GO-TANF has been demonstrated to detect the hepatitis B virus (HBV). For the detection of viral DNAs, the activated CRISPR-Cas12 can cleave the ssDNA to detach RAuNPs from the SERS substrate, weakening the SERS intensity. Without the nucleic acid amplification, the spectra of SERS signals at different viral concentration were collected and the intensities at the Raman shift of 1656 cm^{-1} were plotted towards viral concentrations (**Figure 2-10b-c**). The detection limit of viral DNA was 1 aM with a dynamic range from 1 aM to 100 pM. In addition, the authors further developed a nanoarray for the multiplexed detection of three viruses, including HBV, human papillomavirus 16 (HPV-16), and HPV-18.

Nanopore sensors. Rapid advances in nanopore sensors have led to significant improvements in DNA sequencing. In a typical nanopore sensor, charged DNAs are electrophoretically driven through a nanoscale orifice to block the passage of ions, resulting in a dip in the current.⁴ Each tip in the current indicates a reading event through the nanopore. As shown in **Figure 2-10d**, an abundance of the nanopore reading events was observed for circular ssDNA reporters without the *trans*-cleavage from CRISPR/Cas12 system. However, the degradation of ssDNA reporters by the activated CRISPR/Cas12 system caused a significant reduction of reading events. To characterize the detection specificity, the authors designed two sets of HIV-1 DNA targets and assays, in which each assay was specific to its target (Assay 1 - Target 1 and Assay 2 - Target 2). As shown in **Figure 2-10e**, reading events were significantly reduced for target 1 in assay 1 and target 2 in assay 2, indicating the CRISPR-based nanopore sensors have a high detection specificity.

2.6 Conclusion

The molecular diagnosis has been greatly extended by the discovery of CRISPR systems, and greatly benefited by the cleavage mechanisms possessed by different CRISPR Cas nucleases. The integration of nucleic acid amplification methods with the CRISPR system is crucial for the early detection and prevention of diseases, as it enables the amplification of DNA and RNA from even trace amounts. This capability is essential for accurate and sensitive detection in various scenarios, such as environmental monitoring, food safety, and clinical diagnostics. Furthermore, different signal transduction models, such as colorimetry, fluorescence, or electrochemistry, can be employed to provide the desired detection sensitivity, dynamic range, and compatibility with the

intended detection platform or device. This adaptability allows the CRISPR-based biosensors to have diverse applications, from rapid in-field detection to high-throughput diagnostics.

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Chapter 3

CRISPR/Cas9-mediated genome editing of T4 bacteriophage for high-throughput antimicrobial susceptibility testing

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3.1 Abstract

The accurate and effective determination of antimicrobial resistance is essential to limit the spread of infectious diseases and ensure human health. Herein, a simple, accurate, and high-throughput phage-based colorimetric sensing strategy was developed for antimicrobial susceptibility testing (AST). Taking advantage of the CRISPR/Cas9 system, the genome of T4 phage was modularly engineered to carry lacZ-alpha (lacZa), a marker gene encoding the α -fragment of β -galactosidase (β -gal). T4lacZa phages were identified by blue-white selection and then used for a biosensing application. In this strategy, the bacterial solution is exposed to T4lacZa phage, causing target bacteria to overexpress β -gal. Upon the addition of a colorimetric substrate, the β -gal initiates an enzymatic reaction, resulting in a solution color change from yellow to red. This sensing strategy offers a visual way to monitor bacterial growth in the presence of antibiotics, enabling the determination of bacterial antimicrobial susceptibility. As a proof-of-concept, our developed sensing strategy was successfully applied to identify 9 different multidrug-resistant *Escherichia coli* (E. coli) in urine samples with 100% specificity. Compared with conventional disk diffusion susceptibility tests, the engineered phage-based sensing strategy can shorten the detection time by at least half without losing the detection sensitivity, providing an alternative high-throughput method for AST in clinical diagnosis.

Keywords: CRISPR/Cas9, engineered bacteriophage, antimicrobial susceptibility testing, clinical sample

3.2 Introduction

The emergence of antimicrobial-resistant bacteria posed a great threat to human health. According to statistics from the Centers for Disease Control and Prevention (CDC), there are over 700,000 deaths per year attributed to antimicrobial resistance.⁵⁻⁷ It is predicted that the number of deaths may increase to 10 million by 2050 if no effective intervention is taken.⁸⁻¹⁰ In response to the critical challenge, antimicrobial susceptibility testing (AST) has emerged as an indispensable tool. AST not only facilitates the accurate identification of antimicrobial-resistant bacteria but also enables healthcare providers to tailor antibiotic therapies effectively. Moreover, AST is critical to the development of comprehensive public health strategies aimed at mitigating the spread of antimicrobial-resistant bacteria and infectious diseases. Consequently, advancing technologies and innovation in AST remain imperative to effectively address the evolving challenges of antimicrobial resistance.

Currently, conventional culture-based methods are regarded as the gold standard of AST, including broth dilution, disk diffusion, and epsilometer test (E-test). The principles of these methods are mainly based on the phenotypic response of bacteria toward different antimicrobial drugs. Even though the culture-based methods provide a straightforward way to determine the viability of bacteria during drug exposure, they are limited by time-consuming incubation. These methods typically take 16-24 hours from sample to result, which is not conducive to urgent antibiotic therapy. As alternatives, some genotypic methods are developed to detect known resistance genes without the need for incubation, such as polymerase chain reaction (PCR), loop-mediated isothermal amplification (LAMP), and rolling circle amplification (RCA).¹²⁻¹⁴ The genotypic methods greatly shorten the detection time compared to culture-based methods. However, the lack of a universal resistant mechanism and the huge genetic variability hinder the widespread applications of genotypic methods. For example, there are more than 800 resistance genes for β -lactam class antibiotics alone.¹² To achieve rapid and sensitive detection, some research developed impedance-based microfluidic platforms for AST.¹⁵⁻¹⁷ For instance, microfluidic impedance cytometry has been constructed to analyze individual bacteria following antibiotic exposure, where the measured electrochemical signals reflect the phenotypic response of bacteria to specific antibiotics.¹⁵ This approach reduces detection time to around 30 minutes, significantly faster than conventional methods. However, the limited number of electrochemical stations and the high cost of electrodes pose challenges for high-throughput AST using electrical

impedance-based techniques. To effectively combat multidrug-resistant bacteria, a high-throughput AST method that leverages the phenotypic response of bacteria to antimicrobial drugs would be highly beneficial for practical applications.

Recently, several studies have utilized engineered bacteriophage (phage) for AST.¹⁸ For instance, the T7 phage was engineered to carry the *lacZ* gene and then used to detect ampicillin-resistant *Escherichia coli* (*E. coli*) strains. Compared with the above-mentioned AST methods, the engineered phage-based method utilizes the phenotypic response of bacteria while shortening detection time. While the engineered phage holds the potential to improve current AST methods, assembling specific genes into the phage genome is still a challenge. For now, the recombination engineering method is the most common approach to generate engineering phage *in vivo*.^{22, 23} The phage genome was assembled with a cytosolic recombination template through homologous recombination. However, the low recombination frequencies (approximately $10^{-8} - 10^{-5}$) make the isolation of engineered phages a very laborious process. As an emerging technology, cell-free transcription–translation (TXTL) has been developed to construct recombinant phages *in vitro*. In this method, PCR is used to amplify overlapping fragments of the phage genome containing the desired mutations. These fragments are then assembled into a complete phage genome using Gibson assembly. The recombinant phage can then be rebooted through *in vitro* transcription and translation within a cell-free expression system, bypassing the need for a live bacterial host for phage propagation. While the TXTL method allows multiplex simultaneous mutations and offers high flexibility in phage genome engineering, its efficiency tends to decrease as the genome size increases. This limitation could pose challenges in engineering the phage with a larger genome or incorporating more complex genetic modifications.²⁸ As an alternative, the clustered regularly interspaced short palindromic repeats (CRISPR) system was developed for engineering the larger phage genome.²⁹ The CRISPR system was designed to recognize and cleave the target region in the phage genome, followed by the homologous recombination between the cleaved phage genome and donor plasmid to generate the recombinant phage. Taking advantage of high programmability and efficiency, the CRISPR system integrated with homologous recombination become a promising method for phage genome editing.

In this study, we developed a CRISPR/Cas9-mediated gene editing platform for phage engineering. The *lacZ*-alpha (*lacZa*) gene was inserted into the T4 phage genome at a high recombination frequency through the CRISPR/Cas9 system. The recombinant phage (T4_{*lacZa*} phage)

could be screened by the blue-white selection and further applied to identify antibiotic-resistant bacteria. The T4_{lacZa} phage-based AST achieves 100% specificity for the identification of 9 different multidrug-resistant *Escherichia coli* (*E. coli*) in urine samples. The results of T4_{lacZa} phage-based AST were compared with those of conventional disk diffusion susceptibility tests. To the best of our knowledge, this is the first study to utilize a CRISPR/Cas9-based platform to engineer phage for AST. The developed platform provides a versatile and efficient way for generating and selecting engineered phages, which have great potential in clinical diagnostic and therapeutic applications.

3.3 Materials and Methods

3.3.1 Bacterial strains, phages, and chemicals

Escherichia coli NEB5 α (*E. coli* NEB5 α) was purchased from New England Biolabs (Ipswich, WA). *Escherichia coli* bacteriophage T4 was purchased from ATCC (Manassas, VA). The T4 ligase, PCR master mix, restriction enzymes, and DNA purification kit were purchased from New England Biolabs (Ipswich, WA). All DNA primers were synthesized by Integrated DNA Technologies (Coralville, IA). The chlorophenol red- β -D-galactopyranoside (CPRG) was purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals were purchased from Fisher Scientific (Hampton, NH).

3.3.2 Plasmid construction

The Cas9 plasmid was constructed by cloning the crRNA protospacer sequence into the pCas9 plasmid (Addgene plasmid #42876). The pCas9 plasmid was digested with the BsaI enzyme first and then ligated with the crRNA protospacer sequence. The crRNA protospacer was designed to target the soc gene of T4 phage with the sequence TGTGAACGTCAGAATAAAGA. The oligoes for the construction of crRNA protospacer were listed in **Table 1**. The ligation products were transformed into *E. coli* NEB5 α chemically competent cells for amplification.

The pCRISPR plasmid (donor plasmid) was constructed using Gibson assembly, which consisted of four parts, the backbone, two homologous arms, and the lacZ-alpha (lacZa) sequence. All primers for the construction of donor plasmid were listed in **Table 1**. The backbone of the donor plasmid was amplified from pCRISPR (Addgene plasmid #42875). The homologous arms were cloned from the T4 phage genome. The lacZa sequence was amplified from the M13KE plasmid (New England Biolabs, Ipswich, WA). All fragments are assembled into the donor plasmid

using Gibson assembly, prior to transformation into *E. coli* NEB5 α chemically competent cells. The sequence of all inserts was confirmed by Sanger sequencing.

Table 3-1 Primers used to construct the donor plasmid

Primer name	Sequence	Amplicon
260_soc_crRNA_F	AAACTGTGAACGTCAGAATAAAGAG	crRNA spacer (target soc)
261_soc_crRNA_R	AAAACCTCTTTATTCTGACGTTTACA	
262_pCRISPR_F	aagtgcgcagaccccgtagaaaagatcaaaggatct	pCRISPR
263_pCRISPR_R	aacaacatcgctcagtggaaacgaaaactcac	backbone
264_T4F_F	cactgagcgatgtgtttcttgatgcgtgaacaaa	homologous arm (left)
265_T4F_R	atcatggatcatgtaattctctttatttaaattacatgattattatacatctcttttc	
266_lacZa_F	ggagaattacatgaccatgattacgccaagct	lacZa
267_lacZa_R	gtccttgagttattaaccaataggaacgccatcaaaaataattcg	
268_T4F2_F	ctattggttaataactcaaggactccttcgggagtc	homologous arm (right)
269_T4F2_R	ggggtctgcgcactttctgccgtaattataaat	

3.3.3 CRISPR/Cas9-based genome editing for T4 bacteriophage

E. coli NEB5 α cell containing both Cas9 plasmid and donor plasmid was used for genome editing of T4 phage. The wild-type *E. coli* NEB5 α cell was used as a control. One single colony of *E. coli* NEB5 α containing Cas9 plasmid and donor plasmid was inoculated into LB media (containing 25 μ g/mL chloramphenicol and 50 μ g/mL kanamycin) and incubated at 37°C overnight. The overnight culture (300 μ L) was mixed with wild-type T4 phage (100 μ L, 10⁴ PFU/mL) and then incubated for 7 min at 37°C. After that, the top agar (3 mL, with 25 μ g/mL chloramphenicol and 50 μ g/mL kanamycin) was added into the bacteria-phage mixture and then poured onto a LB agar plate containing chloramphenicol and kanamycin. The plate was incubated at 37°C overnight. The efficiency of plating was calculated by dividing the number of phages produced from infection of *E. coli* NEB5 α containing both Cas9 plasmid and donor plasmid by the number of phages generated from infection of wild-type *E. coli* NEB5 α . The recombinant phages were identified by blue-white selection and Sanger sequencing.

3.3.4 Transformation One-step phage growth

A one-step phage growth experiment was carried out as previous report.¹⁹ In brief, the *E. coli* NEB5 α (2×10^8 CFU/mL) at log phase was infected with T4 phages (2×10^7 PFU/mL) at 37 °C for 5 min. The bacteria-phage infection mixture was centrifuged (10,000 g, 2 min) and washed with an LB medium to remove the unabsorbed phage. The infection mixture was diluted 10^5 times and incubated at 37°C under an agitation of 250 rpm. The number of infection centers is determined by the amount of phage in the initial diluted mixture. Every 5 min, samples were withdrawn from the infection mixture and measured by plaque assay. Burst size is defined as the amount of progeny phage produced per infection center.

3.3.5 Colorimetric detection of bacteria using T4_{lacZa} phage

The bacteria solutions (10^6 CFU/mL) were added into LB broth and pre-incubated at 37°C for 3 hours with an agitation of 250 rpm. Afterward, the chlorophenol red- β -D-galactopyranoside (CPRG) solution (24 mM) and T4_{lacZa} phage (10^3 PFU/mL,) solution were added and reacted for another 5 hours. The absorbance intensity of different solutions was measured every hour at the wavelength of 573 nm using a Synergy HTX multimode reader (BioTek Instruments, Winooski, VT).

3.3.6 Antimicrobial susceptibility testing (AST) for urine samples

Normal human urine was purchased from BioIVT (Westbury, NY) and filtered by 0.22 μ m pore size membrane. The *E. coli* NEB5 α was transformed with plasmids carrying different antibiotic resistance genes, including the AmpR for ampicillin, KanR for kanamycin, SmR for spectinomycin, CmR for chloramphenicol, and GmR for gentamicin. To simulate the application scenarios, the *E. coli* NEB5 α (1×10^6 CFU/mL) with different antibiotic resistance genes were spiked into the normal human urine sample and distributed into six groups within a 96-well plate. Each group was treated with a different antibiotic: 100 μ g/mL ampicillin, 25 μ g/mL chloramphenicol, 10 μ g/mL gentamycin, 50 μ g/mL kanamycin, or 50 μ g/mL spectinomycin. The sterile water was used as the negative control. The samples were then pre-incubated at 37°C with agitation at 250 rpm for 3 hours. The following steps were the same as the colorimetric detection of bacteria using T4_{lacZa} phage. The wild-type *E. coli* NEB5 α without any antibiotic-resistance gene was used as a positive control.

3.3.7 Disk diffusion susceptibility test

Five different antibiotic disks were prepared by dropping a certain amount of antibiotic (10 µg ampicillin; 5 µg spectinomycin; 5 µg kanamycin; 2.5 µg chloramphenicol; or 1 µg gentamicin) onto the filter paper disk and drying it at room temperature.¹⁹ The fresh cultures of multi-drug resistant *E. coli* NEB5α (200 µL) were plated on LB agar plates individually. The antibiotic disks were then fixed to the surface of LB agar plates using sterile tweezers. The plates were incubated at 37°C for 18 hours. The filter paper disk without antibiotics was regarded as a control.

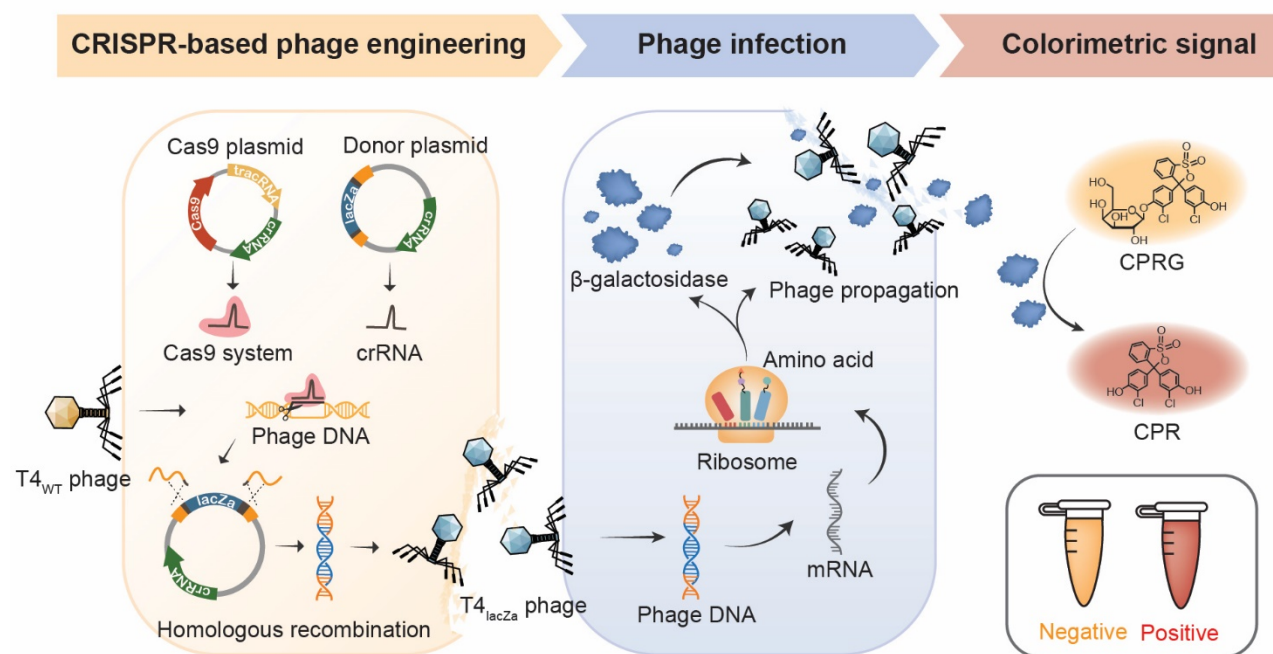


Figure 3-1 Overview of CRISPR/Cas9 mediated genome editing of T4 phage.

1) The T4_{WT} phage is engineered with a *lacZα* gene through the CRISPR/Cas9 system. The *E. coli* strain containing both Cas9 plasmid and donor plasmid express Cas9/sgrRNA complex *in vivo*. During the phage infection, the phage genome is cleaved by the Cas9/sgrRNA complex, and then triggers the homologous recombination between the broken phage genome and homologous arms of the donor plasmid, producing the recombinant phage (T4_{lacZα} phage). 2) The T4_{lacZα} phage is used for further detection of bacteria. The infection of the T4_{lacZα} phages triggers the phage propagation and overexpression of β-galactosidase (β-gal). Subsequently, both T4_{lacZα} phage and β-gal are released upon cell lysis. 3) The detection signal is generated under the enzymatic reaction between β-gal and chlorophenol red-β-D-galactopyranoside (CPRG). The detection results can be determined through the colorimetric signals.

3.4 Results and discussion

3.4.1 Principle of T4 phage genome editing using CRISPR/Cas9 system

As shown in **Figure 3-1**, the principle of CRISPR/Cas9 mediated T4 phage genome editing and the detection of bacteria are illustrated. The wild-type T4 phages (T4_{WT}) are engineered with a lacZa gene through the CRISPR/Cas9 system, generating T4_{lacZa} phages. After that, the T4_{lacZa} phages are used to infect bacteria, resulting in the overexpression of β -galactosidase (β -gal) *in vivo*. Upon cell lysis, the β -gal is released from bacteria and generates the detection signal with the colorimetric substrate.

The CRISPR/Cas9 system consists of two plasmids: Cas9 plasmid and donor plasmid. The Cas9 plasmid includes the coding sequence of Cas9 nuclease, trans-activating CRISPR RNA (tracrRNA) and CRISPR RNA (crRNA) targeting phage genome. The donor plasmid contains the lacZa gene flanked by homologous arms, along with the crRNA cassette. Two plasmids are co-transformed into *Escherichia coli* NEB5 α (*E. coli* NEB5 α) and then express CRISPR system (Cas9:tracrRNA:crRNA complex) *in vivo*. Upon infection with T4_{WT} phage, the CRISPR/Cas9 system recognizes and cleaves the phage genome. This cleavage can be repaired through homologous recombination with donor plasmid, leading to the integration of the lacZa sequence into the phage genome. The recombinant T4_{lacZa} phage is generated and purified for further biosensing applications.

The lacZa gene encodes the α -fragment of β -galactosidase (β -gal), a glycoside hydrolase enzyme commonly utilized as an indicator in enzymatic reactions.³² Consequently, an enzymatic biosensor is developed using the T4_{lacZa} phage. Engineered to function as the sensing element, the T4_{lacZa} phage specifically targets viable *E. coli*. During infection, the T4_{lacZa} phages attach to bacteria and insert their genome into the bacteria cell. Afterward, the inserted DNA takes over bacterial fundamental mechanisms to express the phage particles, inducing the overexpression of β -gal. Following the lysis cycle, the cell membranes are destroyed, releasing the phages and β -gal. In the presence of the colorimetric substrate (chlorophenol red- β -D-galactopyranoside, CPRG), β -gal catalyzes the hydrolysis of CPRG to result in a color change from yellow to red. The absorbance measurement of the reaction solution allows us to derive the colorimetric signal and assess bacterial growth.

3.4.2 Construction of T4_{lacZa} recombinant phage

For the CRISPR/Cas9-mediated gene editing, the Cas9:tracrRNA:crRNA complex functions as a molecular scissor, targeting and cleaving the phage genome. Subsequently, the donor plasmid facilitates the repair of the cleaved genome through homologous recombination. Homologous recombination is a genetic process characterized by the exchange of nucleotide sequences between two DNA molecules that share significant similarity or identity. As shown in **Figure 3-2a**, the *soc* gene is selected as the target for gene editing, wherein it is replaced by the lacZa gene utilizing the CRISPR/Cas9 system. The *soc* gene encodes the small outer capsid protein, a nonessential protein of T4 phage, with approximately 870 copies per capsid.³³ The donor plasmid was designed to contain the lacZa gene flanked by approximately 1000 bp homologous arms derived from the sequence adjacent to the *soc* gene (SegF and Mrh). Using the CRISPR/Cas9 system, the *soc* gene was substituted with the lacZa gene within the phage genome, resulting in the overexpression of β -gal protein during phage infection.

Based on the mechanism of the CRISPR/Cas9 system, three infection scenarios were compared in **Figure 3-2b-d**. Firstly, if the bacteria possess only the Cas9 plasmid, the Cas9:tracrRNA:crRNA complex would cleave the T4_{WT} phage genome upon infection (**Figure 3-2b**). As a result, none of the phages would survive in the plaque assay. Conversely, in the presence of only the donor plasmid, the bacteria would lack resistance to phage infection. All phages could survive after phage infection and most of them retain the wild-type genome (**Figure 3-2c**). In this case, it is important to note that recombinant phages may be generated through homologous recombination between the phage genome and the donor plasmid. Previous research indicates that the efficiency of spontaneous homologous recombination ranges from 10^{-8} to 10^{-5} .³⁴ Finally, if the bacteria contain both the Cas9 plasmid and donor plasmid, the Cas9 system would facilitate the replacement of a targeted phage gene with the lacZa gene from the donor plasmid. In this case, only recombinant phages carrying the lacZa gene would survive in plaque assay (**Figure 3-2d**).

The plaque assay and polymerase chain reaction (PCR) were used to verify the proposed hypothesis. T4_{WT} phages were incubated with *E. coli* NEB5 α processing various plasmid combinations. Subsequently, top agar was added to the mixture and poured on the LB plates. As shown in **Figure 3-2e**, no plaque was observed after the T4 phage infected the bacteria containing only Cas9 plasmid, demonstrating that all phages were unable to survive due to cleavage by Cas9:tracrRNA:crRNA complex. In contrast, when the T4 phage infected the bacteria containing

only donor plasmid, a significant number of plaques were produced. The efficiency of plating was calculated by dividing the number of plaques produced from the bacteria containing various plasmid combinations by the number of plaques produced from the bacteria without any plasmid. It was found that the efficiency of plating was 87.3% during the infection of bacteria containing only donor plasmid (**Figure 3-2f**). Eight plaques were randomly selected from the plate for PCR (**Figure 3-2g**). It is shown that all plaques kept the wild-type genome, which was further confirmed by Sanger sequencing. Furthermore, the plaque assay results were investigated when the T4 phage infected the bacteria containing both Cas9 and donor plasmids. The efficiency of plating is 2.7%,

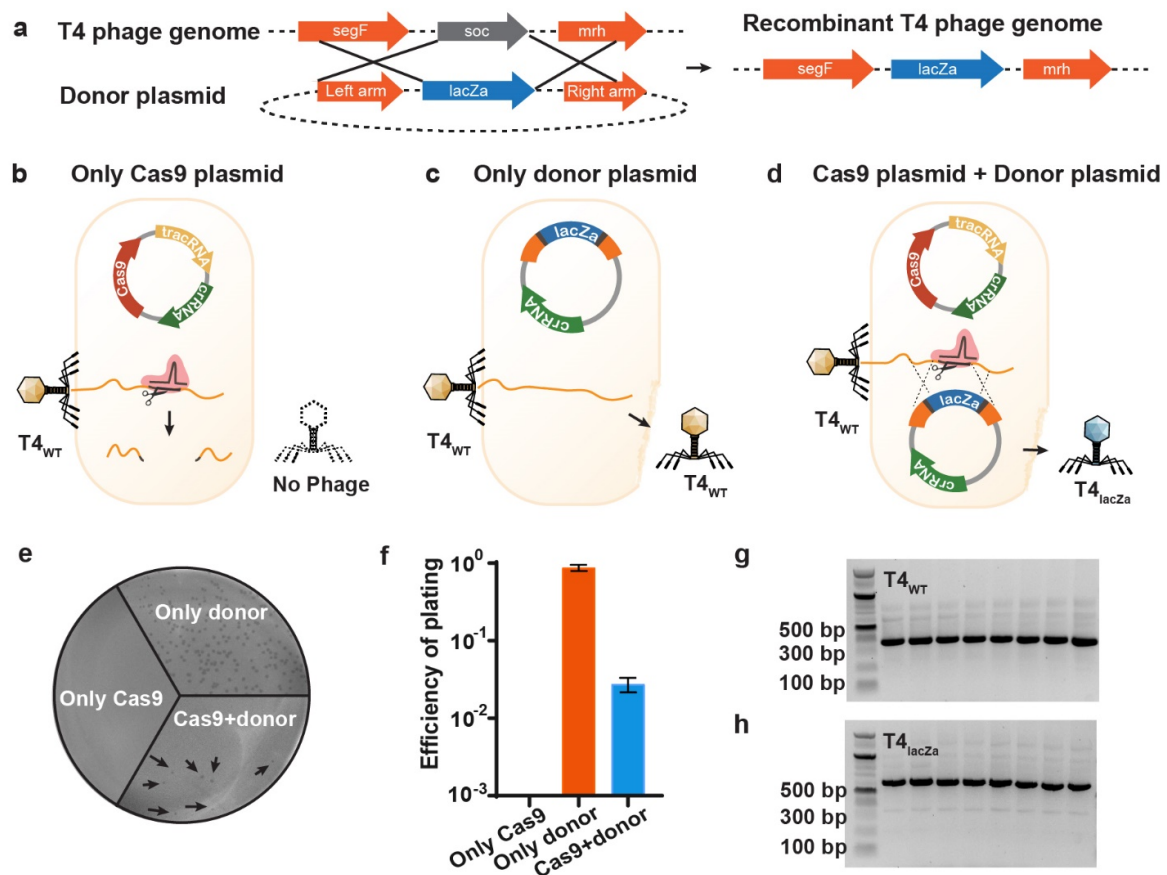


Figure 3-2 Construction of T4_{lacZa} recombinant phages by CRISPR/Cas9 system.

(a) Schematic illustration of homologous recombination. (b) T4_{WT} phage infection of *E. coli* containing only Cas9 plasmid. (c) T4_{WT} phage infection of *E. coli* containing only donor plasmid. (d) T4_{WT} phage infection of *E. coli* containing both Cas9 plasmid and donor plasmid. (e) Phage plaques from various infection scenarios. (f) Efficiency of plating of various infection scenarios. (g) Characterization of phage plaques after the infection with *E. coli* containing only donor plasmid. (h) Characterization of phage plaques after the infection with *E. coli* containing both Cas9 plasmid and donor plasmid.

which is about 32 times lower than the bacteria carrying only donor plasmid. Similarly, the PCR and sequencing results demonstrated that 8 randomly selected phage plaques contain the *lacZa* gene (**Figure 3-2h**). All these results indicate that the T4_{WT} phage was successfully engineered into the T4_{lacZa} phage using the CRISPR/Cas9 system.

3.4.3 Characterization of recombinant phages

Leveraging the unique characteristic of the *lacZa* gene, a blue-white selection was designed to rapidly differentiate the T4_{lacZa} phage from the T4_{WT} phage. The blue-white selection relies on the enzymatic reaction between β -gal and X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside), an organic compound consisting of galactose linked to a substituted indole. In the presence of T4_{lacZa} phage, the overexpressed β -gal hydrolyzes X-gal, releasing the substituted indole. This indole then spontaneously dimerizes, forming an insoluble, intensely blue product in the phage plaque. Using the blue-white selection method, the T4_{lacZa} phage can be visually identified on the X-gal plate (**Figure 3-3a**). The plaques of T4_{WT} phage and T4_{lacZa} phage at different concentrations on the X-gal plate are shown in **Figure 3-3b**. It is observed that all T4_{WT} phages formed clear plaques, whereas all T4_{lacZa} phages produced blue plaques. Subsequently, phage plaques from different infection scenarios were transferred onto the X-gal plates for blue-white selection. In

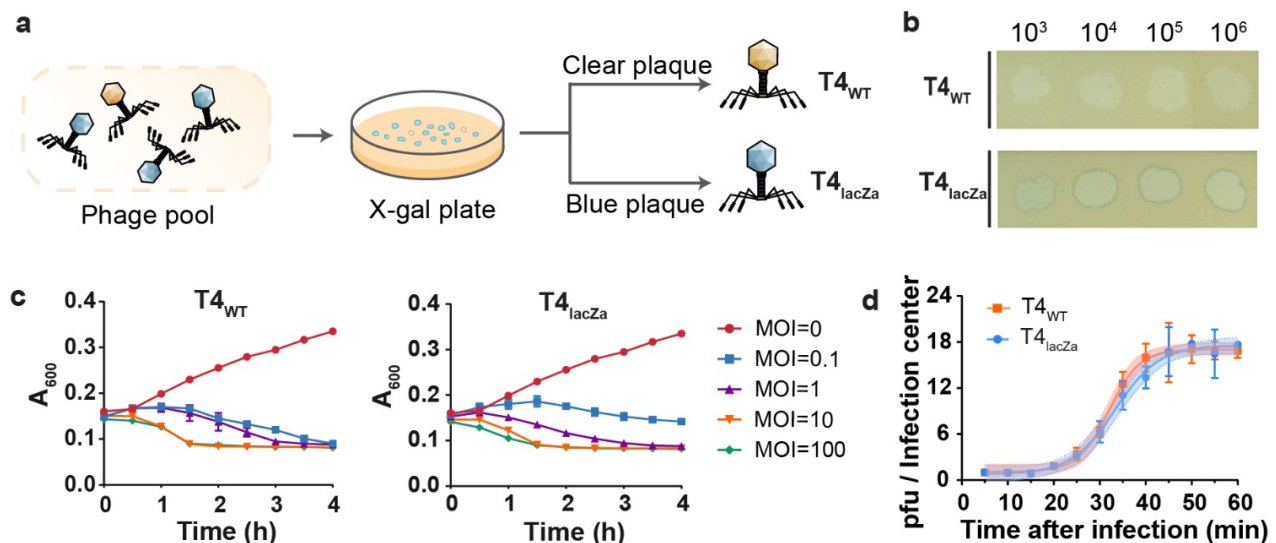


Figure 3-3 Characterization of T4_{lacZa} recombinant phages.

(a) Workflow for blue/white selection of engineered phages. (b) Photograph of blue/white selection for engineered phages. (c) Inhibitory effect of the T4_{WT} and T4_{lacZa} phage on *E. coli* cells. (d) One-step growth curve of T4_{WT} and T4_{lacZa} phage.

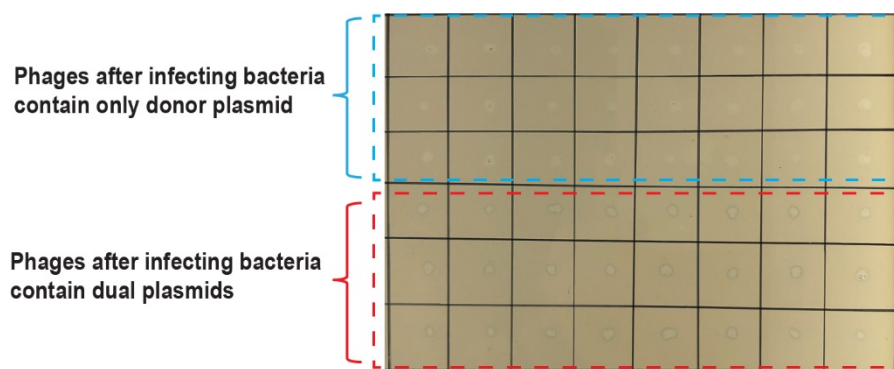


Figure 3-4 The blue-white selection method.

This method is used to identify phages from plaques formed after infecting bacteria containing different plasmids.

each infection scenario, 24 plaques were selected. **Figure 3-4** demonstrated that plaques generated from bacteria containing only donor plasmid exhibit colorless plaques, whereas those picked from bacteria containing dual plasmids (Cas9 plasmid and donor plasmid) appeared blue color, indicating all recombinant phages carried the *lacZa* gene. Therefore, the blue-white selection method is a sophisticated approach that effectively addresses the issues of time-consuming and labor-intensive processes during recombinant phage screening. In addition, although we did not observe any blue plaques when screening phages generated from the bacteria containing only the donor plasmid, there remains a very slight possibility (frequencies range from 10^{-8} to 10^{-5}) that phages could be produced through spontaneous homologous recombination.^{28, 35}

The infection efficiency of T4 phage was then evaluated by the cell density at different multiplicity of infection (MOI), defined as the ratio of phage to host bacteria. Various concentrations of phages were individually mixed with *E.coli* cells (10^8 CFU/mL) and incubated for 4 hours. Absorbance intensities at 600 nm (A_{600}) were recorded every 30 min. As shown in **Figure 3-3c**, the T4_{WT} and T4_{lacZa} phages exhibited similar infection responses. The infection efficiency increased with the higher MOI value. Additionally, a one-step growth curve experiment was performed to compare the infectivity between the T4_{WT} and T4_{lacZa} phages (**Figure 3-3d**). Interestingly, the T4_{lacZa} phage generated a similar burst size of progeny phage as the T4_{WT} phage. These results demonstrated that the *soc* gene is a nonessential gene on the T4 phage genome. Moreover, the engineered phage can incorporate functional protein without losing infectivity, making it an ideal sensing element for biosensing methods.

3.4.4 Colorimetric detection of bacteria using T4_{lacZa} phage

Taking inspiration from previous results, we aimed to design an engineered phage-based biosensor leveraging the unique properties of the T4_{lacZa} phage. As illustrated in **Figure 3-5a**, with the assistance of T4_{lacZa} phage, a colorimetric biosensor was established between viable bacteria and detection signals. Upon attaching to the bacteria, the T4_{lacZa} phage injected its genome into the bacterial cytoplasm, triggering the overexpression of phage particles within the host cell. This process includes the expression of lacZa, the gene encoding β -gal. Following a lytic cycle, progeny phages and β -gal were released from the host cell, initiating the enzymatic reaction with the colorimetric substrate (CPRG). As the galactoside analog, CPRG was degraded into galactose and

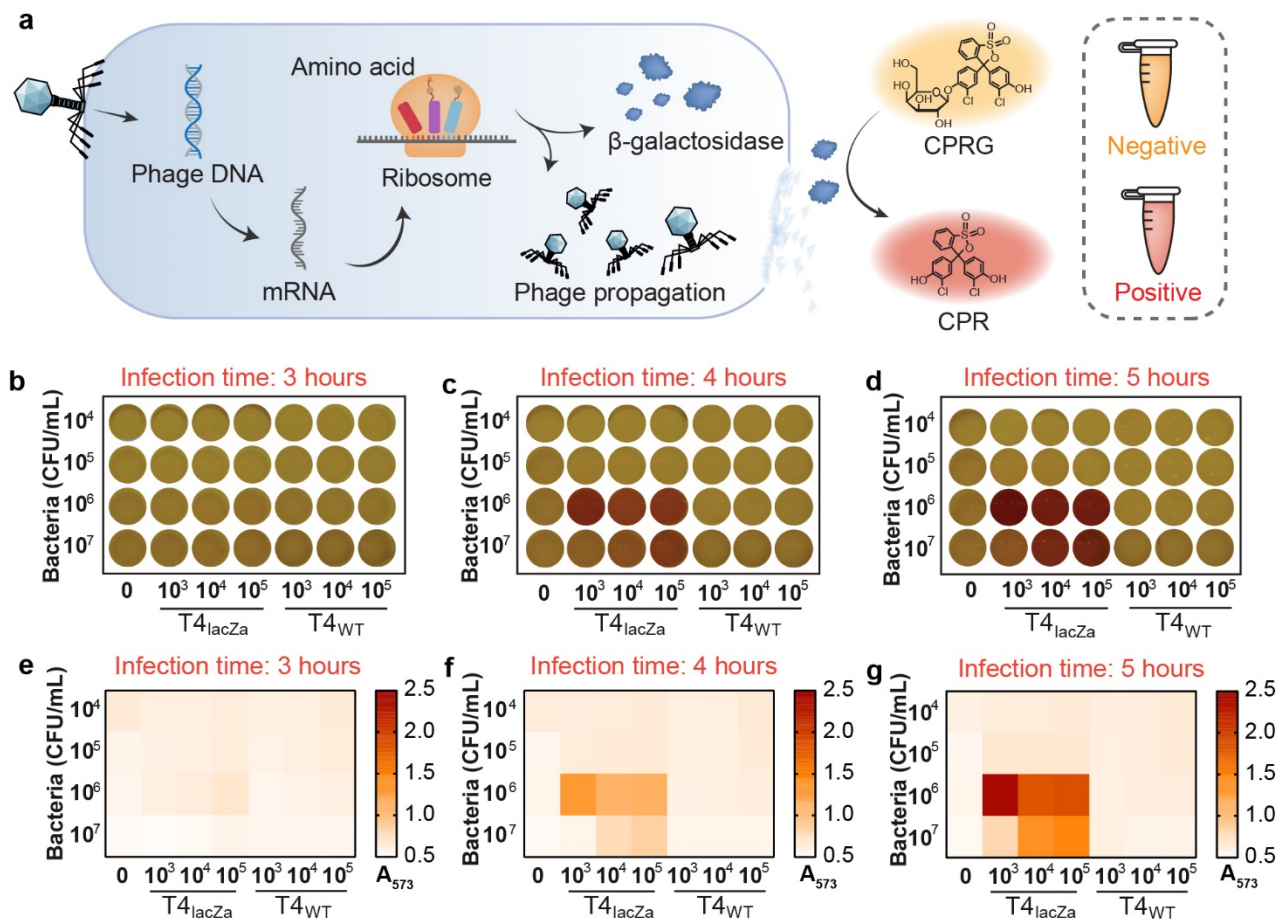


Figure 3-5 Colorimetric detection of *E. coli* using T4_{lacZa} phages.

(a) Schematic illustration of the principle of the colorimetric biosensor for the detection of *E. coli*. Digital photographs of the reaction solution for 3 hours (b), 4 hours (c), and 5 hours (d) of infection. Heat maps of colorimetric response for 3 hours (e), 4 hours (f), and 5 hours (g) of infection. Colorimetric response based on absorbance intensities at 573 nm (A_{573}).

chlorophenol red, leading to a solution color change from yellow to red. The resulting color change serves as a visual indicator of bacterial presence, providing a sensitive and reliable detection method for various applications, including viable bacterial detection and antimicrobial susceptibility testing (AST).

Infection time is one of the main factors that influence the colorimetric results of the engineered phage-based biosensor. The bacteria were pre-enriched at different concentrations in a 96-well plate for 3 hours. Subsequently, the CPRG substrate and phages at different concentrations were added to the bacteria and incubated under agitation. The absorbance at 573 nm (A_{573}) and the photograph of 96-well plates were collected every hour. The T4_{WT} phage is regarded as the control. As the infection time increased, the color of the bacteria-phage solution changed from yellow to red, reaching a dark red after the incubation of 5 hours (**Figure 3-5b-d**). Therefore, the infection time was optimized to be 5 hours.

The concentration of phage and bacteria is the other key factor of the colorimetric biosensor. The phages at high concentrations could rapidly kill all bacteria in early lytic cycles, resulting in a lower colorimetric signal. Conversely, low phage concentration may require more time to infect the bacteria and produce sufficient β -gal for colorimetric detection. Therefore, there is a trade-off between bacteria and phage concentrations. Various combinations of bacteria and phage were investigated during infection to find the optimal balance. The heatmaps in **Figure 3-5e-g** visualized the absorbance of the different bacteri-phage combinations. It was found that the optimized bacteria concentration is 10^6 CFU/mL while the optimized phage concentration is 10^3 PFU/mL.

Antimicrobial susceptibility testing (AST) for urine samples

Since the emergence of multidrug-resistant strains, the treatment of bacterial infections (such as urinary tract infections) with empirical antimicrobial therapies is becoming increasingly challenging. Disk diffusion, a conventional method for antimicrobial susceptibility testing (AST), has been widely utilized to determine susceptibility in acute urinary tract infections. However, its lengthy culturing time (> 16 hours) delays prompt antimicrobial therapy. To shorten the detection time of AST, the engineered phage-based colorimetric biosensor was applied to detect multidrug-resistant bacteria in urine samples.

The workflow of colorimetric AST is depicted in **Figure 3-6a**. In brief, the bacteria were spiked into a urine sample and pre-enriched with antibiotics for 3 hours. Following exposure to antibiotics, the antibiotic-resistant bacteria survive, whereas the antibiotic-susceptible bacteria perish. After

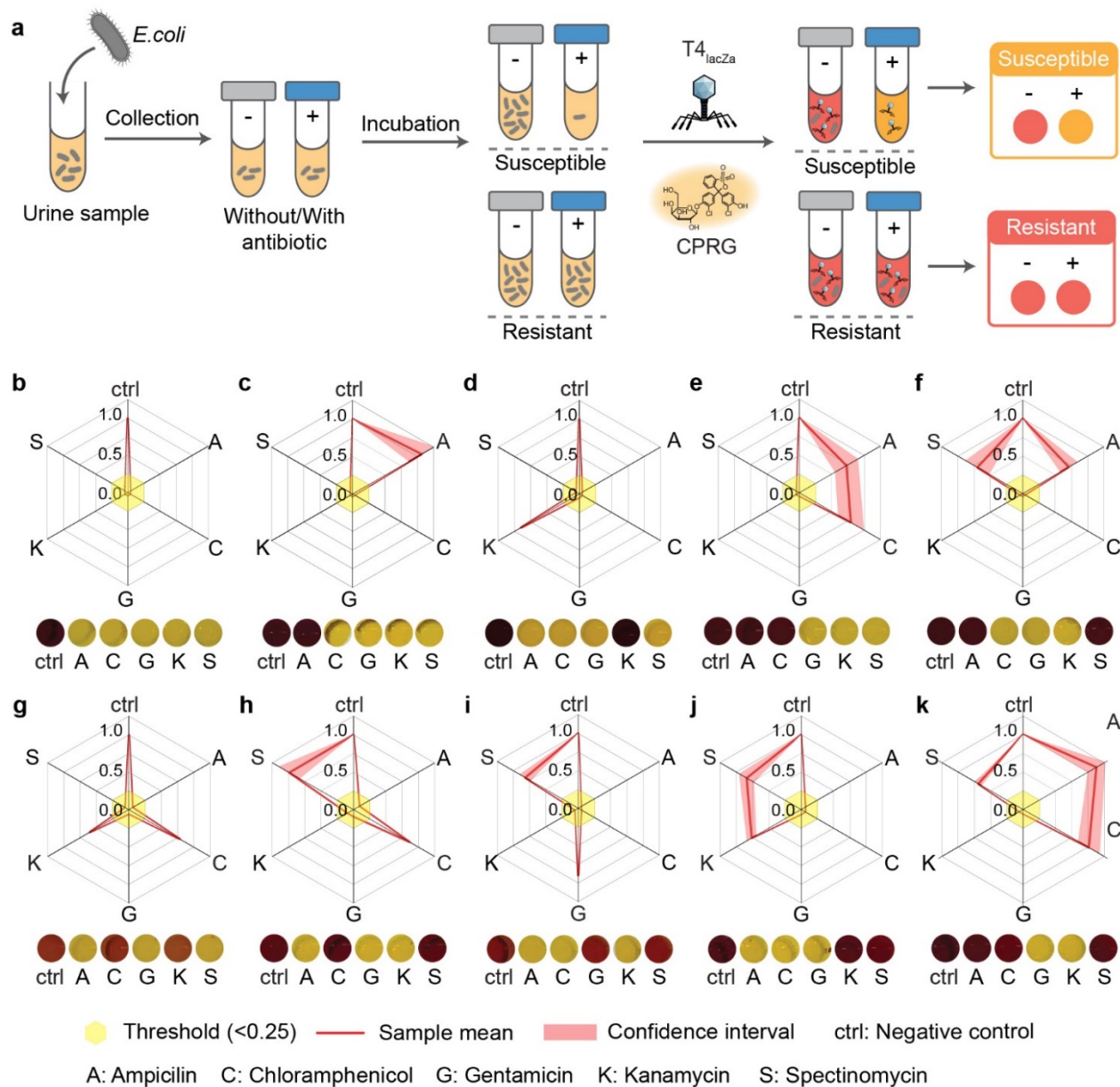


Figure 3-6 High-throughput antimicrobial susceptibility testing using T4_{lacZa} phages.

(a) The workflow of the colorimetric AST in urine samples. (b-k) Photographs and radar chart of antimicrobial susceptibility testing results. The bacteria from left to right: (b) wild-type *E. coli* (positive control), (c) ampicillin-resistant *E. coli*, (d) kanamycin-resistant *E. coli*, (e) ampicillin- and chloramphenicol-resistant *E. coli*, (f) ampicillin- and spectinomycin-resistant *E. coli*, (g) kanamycin- and chloramphenicol-resistant *E. coli*, (h) chloramphenicol- and spectinomycin-resistant *E. coli* (i) gentamicin- and spectinomycin-resistant *E. coli* (j) kanamycin- and spectinomycin-resistant *E. coli* (k) ampicillin-, chloramphenicol- and spectinomycin-resistant *E. coli*. The value is calculated by dividing the A₅₇₃ of the experimental group by the A₅₇₃ of negative control group. The concentration of bacteria was 10⁶ CFU/mL, while the concentration of phage was 10³ PFU/mL. All experiments replicated three times.

that, the T4_{LacZa} phage and CPRG were added to the bacterial sample, triggering the colorimetric reaction. The bacteria grow in the urine sample without any antibiotic as a negative control (the group labeled with “-” in **Figure 3-6a**). Based on this, the susceptibility of bacteria was determined by the different colors between the control and the experiment groups. For susceptible strains, after being incubated with antibiotics, the inactive bacteria would be unable to trigger the color change with the T4_{LacZa} phage and CPRG, resulting in a significant difference in solution absorbance compared to the negative control.

The feasibility of an optimized colorimetric AST was demonstrated for 9 antibiotic-resistant bacteria in urine samples, including 2 single-drug resistant bacteria and 7 multi-drug resistant bacteria. Five antibiotics broadly used in the therapy for bacterial infection (ampicillin, chloramphenicol, gentamicin, kanamycin, and spectinomycin) were chosen to provide different resistance profiles. The sterile water was used as the negative control for each bacterium. Plasmids containing antibiotic-resistant genes were transformed into bacteria to confer resistance. The bacterium without any antibiotic resistance was chosen as a positive control. After the antibiotic exposure and phage-based colorimetric detection, we took pictures and recorded the A₅₇₃ for each reaction solution. The detection signal was calibrated by dividing the A₅₇₃ of the experiment group by the negative control group. To minimize the background noise, the threshold was set to 0.25. Values below 0.25 were interpreted as negative results, indicating the susceptibility of the bacteria to the antibiotic. In contrast, values above 0.25 were classified as positive results, showing the resistance of the bacteria to the antibiotic. The wild-type *E. coli*, which lacks any antibiotic resistance genes, was used as the positive control in the experiment. The radar chart in **Figure 3-6b** showed that the positive control group only has signal response in the absence of antibiotics. In contrast, when the *E. coli* carries the ampicillin-resistant gene, the engineered phage-based biosensor generated significant colorimetric signals in both control channel and ampicillin channel (**Figure 3-6c**). Based on this mechanism, the proposed colorimetric biosensor successfully determined 9 antibiotic-resistant bacteria with 100% specifically (**Figure 3-6d-k**). Furthermore, the disk diffusion method was performed for the same antibiotic-resistant bacteria in the urine samples (**Figure 3-7**). The inhibition radius to antibiotic disks were presented in **Figure 3-8**. Based on the inhibition radius generated through negative control group, the values larger than 1.5 mm indicate susceptibility toward the corresponding antibiotic. The bacterial susceptibility pattern using the disk diffusion method perfectly matched with the previous phage-based results, proving

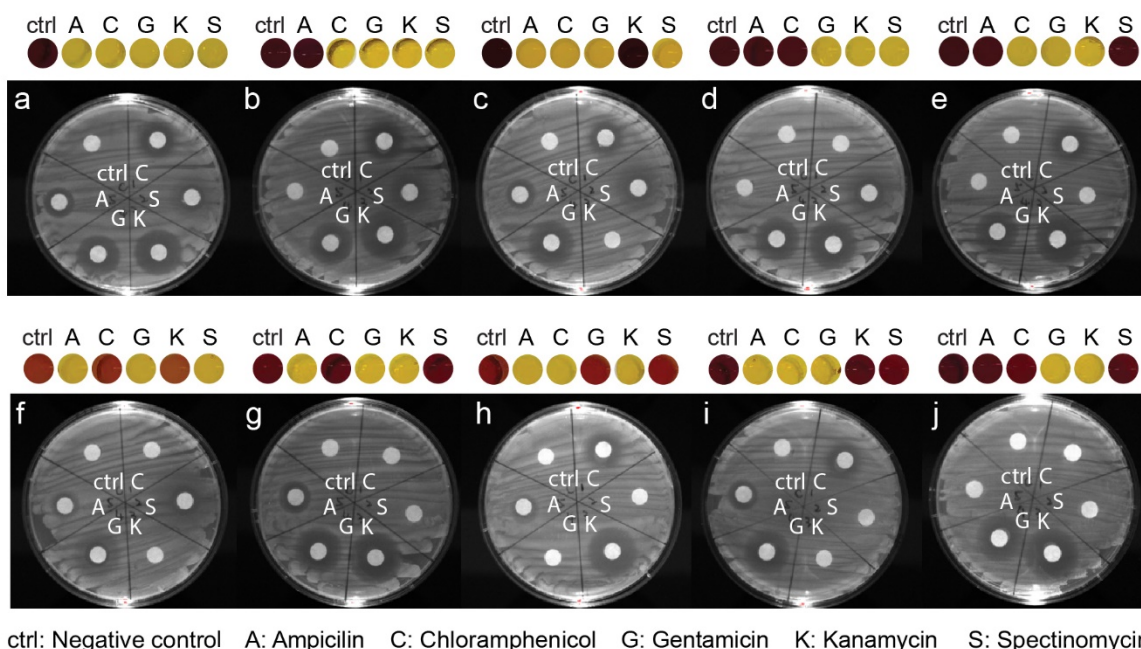


Figure 3-7 Disk diffusion method for AST in urine samples.

(a) The bacteria grown in the urine sample without any antimicrobial was regarded as the negative control. (b) ampicillin-resistant *E. coli*, (c) kanamycin-resistant *E. coli*, (d) ampicillin- and chloramphenicol-resistant *E. coli*, (e) ampicillin- and spectinomycin-resistant *E. coli*, (f) kanamycin- and chloramphenicol-resistant *E. coli*, (g) chloramphenicol- and spectinomycin-resistant *E. coli* (h) gentamicin- and spectinomycin-resistant *E. coli* (i) kanamycin- and spectinomycin-resistant *E. coli* (j) ampicillin-, chloramphenicol- and spectinomycin-resistant *E. coli*.

the reliability of the engineered phage-based colorimetric method. However, there are key differences between the two methods. The disk diffusion method is a signal-off approach, where inhibition zones are formed only when bacteria are sensitive to the antibiotic, resulting in a clear area around the disk. In contrast, our proposed biosensor is a signal-on method, generating a detectable colorimetric signal only when bacteria are resistant to the antibiotic. This makes the signal-on method more straightforward for screening antibiotic-resistant bacteria, as it directly indicates resistance without requiring manual measurement of inhibition zones. Furthermore, the colorimetric signal is visually clearer, eliminating the potential for human error in radius measurement. Importantly, our colorimetric biosensor also reduces the detection time by at least half compared to the disk diffusion method, without compromising specificity, making it a more efficient and user-friendly option for clinic applications.

Although the engineered phage-based detection method has been successfully applied in AST, the sensitivity and efficiency are limited by the expression level of the lacZa gene in the T4 phage. In the future, there are lots of strategies that could be used to address this issue. Firstly, multiplex gene editing could enhance the expression of the lacZa gene. In this study, we chose the soc gene locus for phage gene editing as a demonstration. Another potential target is the hoc gene, which encodes a non-essential capsid protein and is commonly used in phage display. Inserting the lacZa gene at both the hoc and soc loci using the CRISPR/Cas9 system could increase the copy number of the lacZa gene, thereby boosting β -gal production during phage infection. Secondly, optimizing

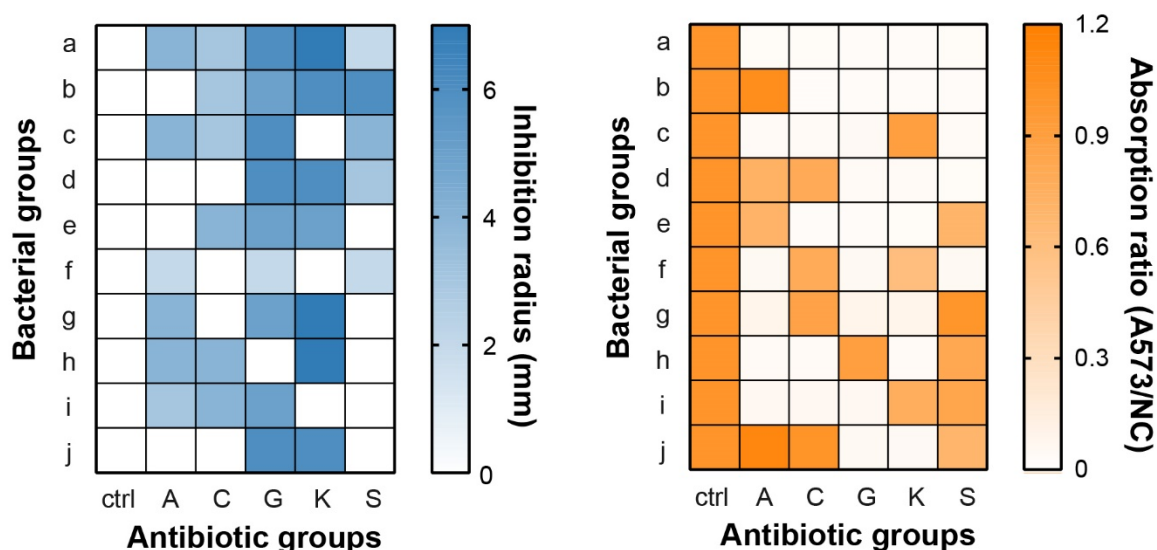


Figure 3-8 The comparison of two methods for AST

The heatmaps of disk diffusion method (left side) and the engineered phage-based colorimetric biosensor (right side) for antimicrobial susceptibility testing in urine samples. Inhibition radius (left) and absorption ratio (right) of different antibiotic resistant *E. coli*. Horizontal axis: (ctrl) negative control, (A) ampicillin, (C) chloramphenicol, (G) gentamicin, (K) kanamycin, (S) spectinomycin. Vertical axis: (a) The bacteria grown in the urine sample without any antimicrobial was regarded as the negative control. (b) ampicillin-resistant *E. coli*, (c) kanamycin-resistant *E. coli*, (d) ampicillin- and chloramphenicol-resistant *E. coli*, (e) ampicillin- and spectinomycin-resistant *E. coli*, (f) chloramphenicol- and kanamycin- resistant *E. coli*, (g) chloramphenicol- and spectinomycin-resistant *E. coli* (h) gentamicin- and spectinomycin-resistant *E. coli* (i) kanamycin- and spectinomycin-resistant *E. coli* (j) ampicillin-, chloramphenicol-, and spectinomycin- resistant *E. coli*. An inhibition radius smaller than 1 mm is classified as resistant. An inhibition radius between 1 to 1.5 mm is considered intermediate. An inhibition radius larger than 1.5 mm indicates susceptibility. The absorption ratio smaller than 0.25 is classified as susceptibility. The absorption ratio larger than 0.25 is regarded as resistant.

Table 3-2 Comparison of the colorimetric results for bacteria without lacZa gene and bacteria containing lacZa gene.

The wells from left to right: negative control, ampicillin, chloramphenicol, gentamicin, kanamycin, and spectinomycin. The infection time is fixed to 5 hours. All experiments replicated three times.

Bacteria resistant gene	Bacteria without lacZa gene	Bacteria contain lacZa gene
ampicillin		
ampicillin, chloramphenicol		
ampicillin, chloramphenicol and spectinomycin		

the reaction process could reduce detection time. Currently, our AST protocol involves a two-step process: bacterial pre-enrichment followed by phage infection. Given that the T4 phage lytic cycle is approximately 30 minutes,^{36, 37} similar to the *E. coli* replication cycle (30-40 minutes), it may be feasible to combine the bacteria, T4 phages, and substrate into a single step, thereby shortening the reaction time. Third, detection efficiency could be enhanced by utilizing alternative phage strains. For instance, the T7 phage, with its simpler replication system and more compact genome, could offer higher protein expression efficiency during infection compared to the T4 phage. Besides of these potential solutions, we also applied the proposed method for the detection of *E. coli* containing lacZa gene. The results were compared with bacteria lacking the lacZa gene (**Table 3-2**). It was found that the bacteria with the lacZa gene generated a signal in 3 hours. When the infection time was fixed at 5 hours, these bacteria exhibited a higher background signal compared to those without the lacZa gene. Therefore, the infection time could be further reduced when applying the colorimetric biosensor to bacteria containing the lacZa gene.

3.5 Conclusion

Taking advantage of high efficiency and specificity, the CRISPR systems are driving a gene editing revolution in different species. In this study, we developed a CRISPR/Cas9-mediated gene editing platform for T4 phage engineering. The engineered phage was equipped with enzymatic markers, making it easy to select from wild-type phage. The innovation of the CRISPR/Cas9

platform breaks down the conventional barriers of phage engineering, offering a versatile and efficient method to customize phages for biosensing applications. Compared to previous CRSIRP-based phage engineering methods,³⁸ our approach is specifically designed for rapid screening of recombinant phages. Using blue-white selection, we can easily distinguish recombinant phages from wild-type phages, simplifying the screening process. Our method provides a more efficient and streamlined solution, addressing the time-consuming and labor-intensive steps often encountered in previous phage engineering workflows. However, the system is not without challenges. For example, the CRISPR/Cas9 system is highly dependent on the PAM sequence. to recognize the target and cleave the target region in the phage genome. Designing crRNA can be difficult if there are few PAM sites available in the target gene sequence. To address this limitation, protein engineering methods, such as directed evolution, could be employed to develop PAM-independent Cas9 nucleases, thereby expanding the capabilities of the CRISPR/Cas9 system and overcoming current constraints.

As a demonstration of feasibility, the engineered phage was applied to antimicrobial susceptibility testing in urine samples, generating a colorimetric signal after 5 hours of infection. Compared to the conventional culture-based AST methods, the engineered phage-based colorimetric biosensor shortens the detection time while maintaining comparable specificity. Furthermore, the liquid-phase reaction facilitates bacteria enrichment and phage-to-bacteria reactions, allowing for simultaneous high-throughput screening of antimicrobial-resistant bacteria. This innovative biosensor holds promise for accurate and efficient detection of viable bacteria, with potential applications in clinical diagnostics and biomedical therapy.

Looking to the future, this work has great potential for applications in a variety of fields. The CRISPR system could be adapted to different phage strains, contributing to phage therapy. Except for the T4 phage, there are lots of phages that have great potential for AST applications, such as the KEP10 and T1 phages. These phages could be used to detect and hinder the growth of *E. coli* during urinary tract infections.² However, these two phages have not been explored for phage engineering due to the time-consuming process of recombinant phage screening. Through changing the crRNA and homologous arms on the Cas9 plasmid and donor plasmid, our proposed CRISPR/Cas9-mediated phage genome editing method could be used to edit different phage genomes and efficiently screen recombinant phages. These engineering phages could be used as a phage cocktail to expand the applicability of the proposed colorimetric biosensor for AST.

Additionally, the engineering phage-based biosensing method could be extended to the minimum inhibitory concentration (MIC) assay. The MIC assay is used to determine the lowest concentration of an antimicrobial agent that prevents the visible growth of bacteria.⁴ Traditionally, MIC assays rely on assessing bacterial turbidity after overnight culture under antibiotic exposure, but our biosensor could offer a faster alternative by using colorimetric signals. Integrating smartphone-based color analysis could further enable portable, on-the-spot detection, making this method more efficient and practical for widespread use.

3.6 References

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Chapter 4

An electrochemical sensing method based on CRISPR/Cas12a system and hairpin DNA probe for rapid and sensitive detection of *Salmonella Typhimurium*

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4.1 Abstract

Salmonella Typhimurium (*S. Typhimurium*) is regarded as a major cause of foodborne diseases, which has been identified as a server threat to public health. Herein, an electrochemical sensing method was developed to detect *S. Typhimurium* in combination with polymerase chain reaction (PCR) and CRISPR/Cas12a (E-CRISPR biosensor). The target DNA extracted from the bacteria was amplified by PCR first, followed by the second amplification through the activation of collateral cleavage activity of Cas12a against signaling hairpin DNA probes, causing the release of electrochemical labels and a dramatic current decrease. The design of hairpin DNA on the electrode also reduced the steric hindrance effect for better Cas12a collateral cleavage efficiency, leading to improved detection performance. Under the optimized conditions, the proposed E-CRISPR biosensor allowed the sensitive detection of *S. Typhimurium* in a linear range from 6.7×10^1 to 6.7×10^5 CFU/mL with a limit of detection of 55 CFU/mL in pure culture. In addition, the E-CRISPR biosensor enables the detection of *S. Typhimurium* in spiked poultry meat with excellent accuracy and sensitivity. This method-CRISPR biosensor has the potential to provide an alternative way for the detection of foodborne pathogens in the food supply chain.

Keywords: CRISPR; hairpin DNA; DNA detection; Electrochemical biosensor; *Salmonella Typhimurium*; food safety

4.2 Introduction

The traditional method to detect *Salmonella* is based on culture-based colony counting, which is sensitive and accurate. However, it is labor-intensive and time-consuming and often takes a few days from sample to result ⁵. As an alternative, polymerase chain reaction (PCR) has the advantages of rapidity and simplicity ⁶, which has been regarded as one of the most promising methods for the rapid detection of *Salmonella* ⁷. For example, Barreda-García's group developed an electrochemical biosensor for sensitive detection of *Salmonella* by combining PCR amplification with a sandwich-type genosensor, which could improve the limit of detection (LOD) of the standard PCR by two orders of magnitude ⁸. Although PCR-based electrochemical biosensors are sufficiently sensitive and specific for detection, they generally require tedious procedures to form “capture probe-target DNA-signal probe” sandwich structures or complex strategies to amplify signals⁹, precluding their application in rapid detection. Therefore, simple and efficient methods for target recognition and signal amplification would enhance the performance of electrochemical biosensors and broaden their applications for in-field detection of target bacteria.

Clustered regularly interspaced short palindromic repeats (CRISPR)-based detection, derived from a gene-editing technology, has shown promising accuracy in nucleic acid detection. The CRISPR/Cas system was discovered in bacteria and archaea, providing acquired immunity against viruses and plasmids by targeting and cleaving the foreign genes ¹⁰. Under the guidance of RNA, Cas protein can accurately recognize the specific gene with high efficiency, opening a new window for elaborating new strategies for biosensing. In recent years, CRISPR-based DNA biosensor has been developed as a very effective tool for the detection of nucleic acids ^{11, 12}. For example, Zhang's group developed a Cas13a-based molecular detection platform (SHERLOCK) to detect DNA and RNA, offering a simple and specific analysis of Zika virus and pathogenic bacteria ¹³. Later, Doudna's group reported a Cas12a-based detection system (DETECTR) for molecular diagnostics of human papillomavirus with attomolar sensitivity ¹⁴. In addition, some studies have integrated CRISPR assays onto the chip to improve the sensitivity and portability of the assay ¹⁵. Zhang's group explored the on-chip amplification-free detection of SARS-CoV-2 using CRISPR/Cas13a and graphene field-effect transistor ¹⁶. Overall, with the emergence of CRISPR-based biosensors, one of the trends is to achieve integration with other methods to improve the sensitivity and accuracy ¹⁷⁻¹⁹.

Taking advantage of miniaturization, low cost, high sensitivity and selectivity, electrochemical technology offers an alternative way of DNA detection. Recently, Liu's group developed a CRISPR-based electrochemical biosensor (E-CRISPR biosensor) to detect human papillomavirus and parvovirus with picomolar sensitivity²⁰. Later on, Cheng's group reported a hairpin DNA (hpDNA) signal reporter to improve the Cas12a cleavage capacity for E-CRISPR, and the E-CRISPR could detect target DNA with high accuracy²¹. Despite significant progress to date, there are still some obstacles to applying E-CRISPR in the food supply chain. First, most E-CRISPR biosensors chose synthetic DNA sequences as targets, which was difficult to simulate the actual application scenarios. For example, in food safety detection, the extraction efficiency of DNA may be significantly interfered with by biological matrices. Furthermore, the concentration of ions (such as K^+ , Na^+ , Mg^{2+}) in the biological matrix can inhibit enzyme activity, and the protein may reduce the sensitivity of the electrochemical interface because of non-specific adsorption. Second, the E-CRISPR biosensors rarely combine with DNA amplification technology, leading to unsatisfactory recognition and signal generation efficiency in complicated samples. Thus, there is a strong impetus for an accurate and stable DNA amplification method that can reduce the food matrix effect and improve sensitivity, expanding the application of the E-CRISPR in the field of biological detection.

In this study, a PCR-incorporated E-CRISPR biosensor is developed for bacteria detection. To achieve sensitive, accurate, and rapid detection, the detection method of this system is based on the following aspects: By incorporating the high amplification efficiency of PCR to bacterial genes and the accurate recognition of target DNA based on the CRISPR method, the E-CRISPR system was gifted with both significant specificity and sensitivity. Moreover, hpDNA is utilized to improve electron transfer rate and reduce the steric hindrance effect on electrodes, providing further enhanced electrochemical performance in target DNA. Using the above-mentioned detection principles, the E-CRISPR biosensor presented the LOD of *S. typhimurium* down to 55 CFU/mL in pure culture, as well as satisfactory performance for poultry meat samples. The proposed biosensor paves a new way to detect foodborne pathogens and represents a significant advancement in the sensitive genetic detection of bacteria for applications in food safety.

4.3 Materials and Methods

4.3.1 Materials

All DNA and RNA oligonucleotides were synthesized by Sangon Biotech (Shanghai, China). RNase inhibitor was purchased from TIANGEN Biotech (Beijing, China). Cas12a were purchased from New England Biolabs (Ipswich, USA). KOD One™ PCR Master Mix was purchased from TOYOBO Biotech (Osaka, Japan). 6-Mercapto-1-hexanol (MCH), and tris(2-carboxyethyl)-phosphine hydrochloride (TCEP) were purchased from Sigma-Aldrich (St.Louis, USA). Other reagents used in this study were purchased from Thermo Fisher Scientific (Waltham, MA, USA). All chemical reagents were analytical grade, and RNase-free water was used throughout this study.

Salmonella Typhimurium (*S. Typhimurium*, ATCC 14028), *Escherichia coli* O157:H7 (*E. coli* O157:H7, ATCC 43888), *Listeria monocytogenes* (*L. monocytogenes*, EGDe), and *Staphylococcus aureus* (*S. aureus*, ATCC 25923) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The clinical isolate of *Vibrio parahemolyticus* (*V. parahemolyticus*, KP9) was provided by Zhejiang Center for Disease Control and Prevention (Hangzhou, China).

CHI660E electrochemical workstation (Shanghai Chenhua Instrument Co. Ltd., China) was used for electrochemical experiments. The working electrodes were gold electrodes with a diameter of 3 mm, the reference electrode was a KCl-saturated calomel electrode (SCE), and the counter electrode was a carbon rod.

4.3.2 Bacterial culture

The stock cultures of *S. Typhimurium* were grown at 37 °C for 16-20 h in Brain heart infusion (BHI) broth. The bacterial cells were diluted 10-fold into the required concentrations after being washed three times by centrifugation at 3,000 rpm for 5 min. To determine the colony-forming unit (CFU) of *S. Typhimurium* concentration, the bacteria were incubated in xyloselysine-tergitol 4 (XLT4) agar plates at 37°C for 18-24 h. Finally, the bacteria were lysed in boiling water for 10 min and kept at 4°C for future studies.

4.3.3 Preparation of hpDNA-modified electrode

The gold electrode was polished according to the reported protocol²¹. The thiolated hpDNA was dissolved in the Tris-HCl buffer (100 mM, pH 7.4) containing NaCl (500 mM), MgCl₂ (5 mM), and TCEP (10 mM). After incubation in the dark at 25°C for 1 h, hpDNA (10 μL, 1 μM) was

then dropped onto the gold electrode surface and incubated overnight at 4°C. To eliminate non-specific adsorption, the electrode was rinsed with PBS (10 mM, pH 7.4) and incubated with MCH (10 L, 1 mM) at 25°C for 2 h. Finally, the electrode was rinsed with PBS (10 mM, pH 7.4), and stored at 4°C. The electrode after each modification was characterized using cyclic voltammetry (CV) in 1 mM $K_3Fe(CN)_6 / K_4Fe(CN)_6$. The schematic of the electrode modification process is shown in **Figure 4-1**.

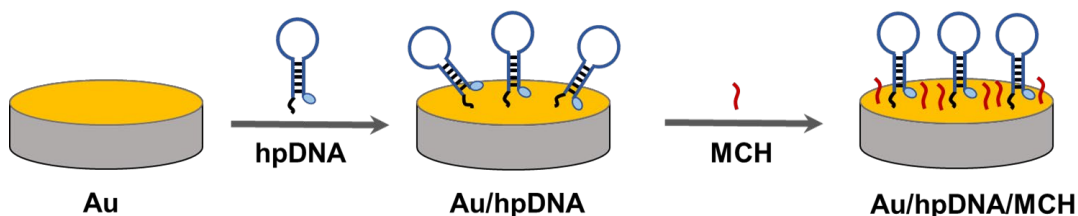


Figure 4-1 Schematic of the electrode modification process

4.3.4 PCR amplification

Forward primer (CTATTGTCACCGTGGTCCAGTTTATCGTTATTA) and reverse primer (CCAATAAAGTTCACAAAGATAATAATGATGCC) were used for the PCR-based amplification of *invA* gene. The total reaction volume (30 μ L) was made up of PCR Master Mix (15 μ L), each primer (1 μ L), temple DNA (1 μ L), and sterile water up to 30 μ L. All reaction components were prepared on ice and quickly transferred to a thermocycler. PCR was performed using the procedure: 98°C for 3 min to initial denature the DNA, cycling (32 cycles) of 98°C for 10 s to denature the DNA, 57°C for 5 s to anneal the primer, 68°C for 5 s to extend the annealed primers. The PCR product was cleaned using TIANquick Midi Purification Kit (TIANGEN Biotech, China).

4.3.5 E-CRISPR detection of bacteria

The E-CRISPR detection of bacteria included the Cas12a-mediated cleavage and electrochemical detection. For the Cas12a-mediated cleavage, 10 μ L of PCR product was mixed with 40 μ L premixed reagent (300 nM crRNA, 200 nM Cas12a, 25 U of RNase inhibitor, and 1 $\times$ NEBuffer2.1) and incubated for 10 min at 37°C. Afterward, the solution was dropped onto the surface of the hpDNA-modified electrode and incubated for 40 min at 37°C. Before electrochemical detection, the electrode was rinsed with PBS three times. In all experiments, the bacterial concentration is the concentration of bacteria in the final reaction buffer.

To measure electrochemical response, differential pulse voltammetry (DPV) was applied before and after the Cas12a-mediated cleavage. It was performed in 10 mM PBS with a pulse width of 0.05 s, a sampling width of 0.017 s, a potential amplitude of 0.05 V, and a pulse period of 0.5 s.

Three terms were used to evaluate the electrochemical signal: current, current ratio (R_i), and response efficiency. Firstly, the current was the pristine electrochemical signal, which can be output directly by the electrochemical station. Secondly, the R_i was regarded as a normalization algorithm, which can remove the variation between different electrodes and show the sensitivity of electrodes. The R_i was determined as a ratio of the current according to the equation: $R_i = (i_0 - i_1)/i_0 \times 100\%$, where i_0 and i_1 are the DPV current of hpDNA modified electrode before and after cleavage, respectively. Thirdly, the response efficiency was regarded as the signal-to-background ratio, which was calculated as a ratio of the R_i of the experiment group (sample with bacterial DNA) to the R_i of the blank group (sample without bacterial DNA), according to the equation: $\text{response efficiency} = R_1/R_0 \times 100\%$, where R_1 and R_0 are the R_i of experiment group and blank group, respectively.

4.3.6 Preparation of poultry samples

According to the references, the addition of target bacteria in the food sample can effectively assess the matrix effect on the sensitivity of the proposed biosensor²²⁻²⁴, which helps to demonstrate the feasibility of the biosensor in practical application. The chicken breast, which was purchased from a nearby grocery store (Hangzhou, China), was used in all real sample experiments. Chicken meat (25 g) was mixed with PBS (225 mL) in filter bags and stomached with a blender for 1 min. Afterward, the chicken solution was collected and spiked with *S. Typhimurium* at concentrations ranging from 10^1 - 10^6 CFU/mL. Finally, these solutions were used for the E-CRISPR detection of *S. Typhimurium* in poultry meat samples. The sample without inoculating *S. Typhimurium* was used as a negative control.

4.4 Results and Discussion

4.4.1 Principle of the PCR-based E-CRISPR method for the detection of *S. Typhimurium*

The principle of the PCR-based E-CRISPR biosensor is illustrated in **Figure 4-2**. The CRISPR system can detect and cleave *invA* genes amplified from the genome of *S. Typhimurium*. As electrochemical signal reporters, the hairpin DNA (hpDNA) probes labeled with methylene

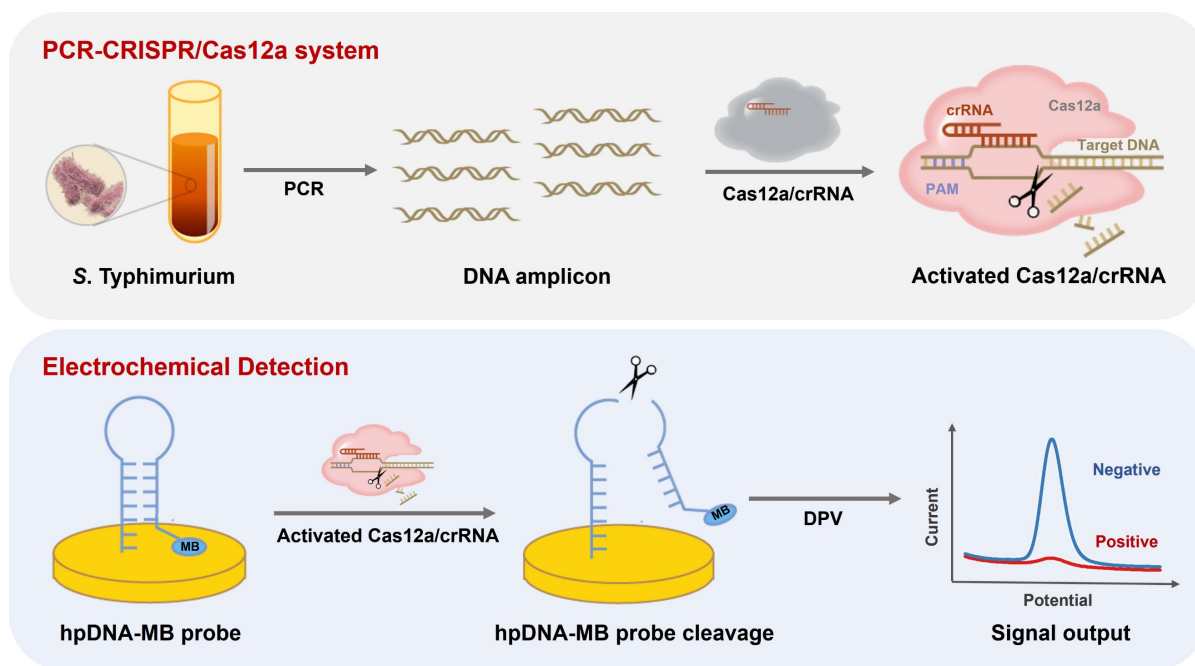


Figure 4-2 Illustration of PCR-based E-CRISPR biosensor.

blue (MB, 3'-terminal) and thiol group (5'-terminal) are modified on the gold electrode surface. Due to the stem-loop structure of hpDNA, MB locates closely to the electrode surface, generating an obvious electrochemical characteristic signal. To ensure high sensitivity of the E-CRISPR biosensor, a PCR assay was used to amplify the target DNA.

In the presence of target DNA, the collateral cleavage activity of the Cas12a protein can be activated, resulting in the indiscriminate cleavage of hpDNA on the electrode. The stem-loop structure of hpDNA is damaged into short fragments, dissociating MB from the electrode surface and thus leading to a dramatic decrease of the electrochemical signal. In the absence of target DNA, the nonactivated Cas12a cannot cleave the hpDNA, resulting in a negligible change of the electrochemical signal. By measuring the redox peak before and after the cleavage of the CRISPR/Cas12a system, the proposed electrochemical biosensor can achieve a specific and sensitive detection of *S. Typhimurium*.

4.4.2 Feasibility of the PCR-based E-CRISPR method

To establish the PCR-based E-CRISPR biosensor, it is important to design the crRNA for CRISPR/Cas12a system. According to the previous study²⁵, the target DNA sequence should contain a T-rich protospacer adjacent motif (PAM) site (TTTN). In this study, *invA* gene was

chosen as a *Salmonella*-specific DNA sequence, and the website (<http://crispor.tefor.net>) was used to design crRNA. As shown in **Figure 4-3a**, the crRNA can target the 23 bp regions of the *invA*.

Because of the high sensitivity, PCR was used to amplify the *invA* gene containing the CRISPR target region. The PCR products can be recognized and cleaved by Cas12a. As shown in **Figure 4-3b**, the 275 bp-long DNA product was observed in the agarose gel image. In addition, the PCR products were further confirmed using Sanger sequencing. These results show that the target DNA sequence was successfully amplified using PCR. Compared with the isothermal amplification techniques (such as loop-mediated isothermal amplification, and recombinase polymerase amplification), PCR displayed a larger linear dynamic range and higher reproducibility. A small quantity of DNA can be amplified as much as 1 million-fold through PCR process ²⁶.

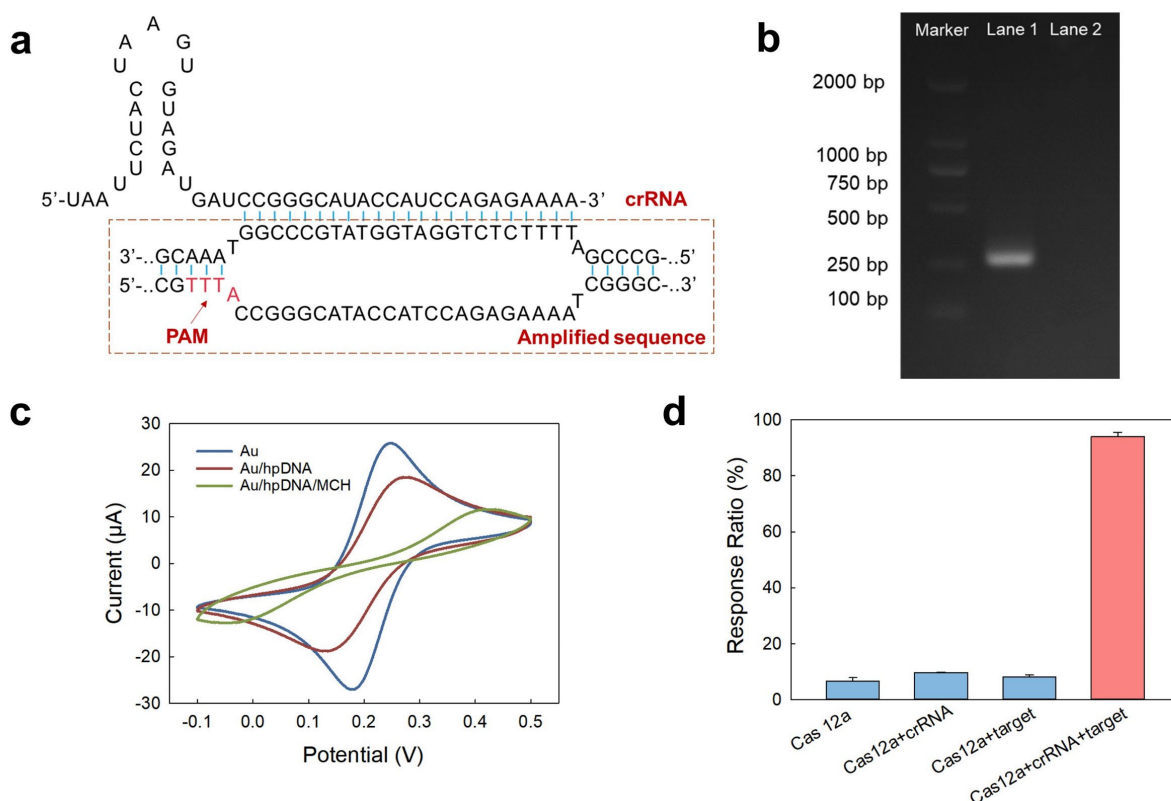


Figure 4-3 Feasibility analysis of the proposed electrochemical sensing method.

(a) The sequence of crRNA for the target site. (b) Characterization of the PCR product of *S. Typhimurium* using agarose gel electrophoresis: lane 1, PCR product with *S. Typhimurium*; lane 2, negative control. (c) CV curves during the electrode modification in 1 mM $\text{Fe}_3(\text{CN})_6/\text{Fe}_4(\text{CN})_6$. (d) Comparison of electrochemical response of E-CRISPR system in the absence of crRNA or target DNA. Error bars were calculated in three independent experiments. Statistical significance was shown as follows: ** $P < 0.01$, target signal vs. control.

Besides, some reports show that combining electrochemical biosensors with PCR can improve the sensitivity of detection a hundred-fold, achieving the single molecule detection⁸. Therefore, PCR has the potential to become a powerful tool for signal amplification in E-CRISPR biosensors.

To characterize the performance of the E-CRISPR biosensor, CV was used to determine the mass transfer efficiency of the gold electrode during modification. After the modification of hpDNA on the gold electrode, the potentials difference (ΔE_p) between the anodic and cathodic peaks increased from 68 mV to 135 mV (**Figure 4-3c**), indicating that the hpDNA impeded interfacial charge transfer because of the negatively charged phosphate backbone. After the MCH blocked the active sites of the electrode, the ΔE_p increased up to 473 mV. In addition, DPV was applied to evaluate the redox peak change after the MCH treatment. When the electrode was treated with MCH, although the redox peak of MB decreased from 1.0 μ A to 0.7 μ A, the baseline of the DPV curve was more stable. This phenomenon can be explained that the 6-carbon chain in MCH promoted the directional arrangement of hpDNA, made them stand up from the surface and reduced the non-specific adsorption of hpDNA²⁷. These results indicate the successful modification of the electrode with hpDNA and MCH. Moreover, the modification of the electrode is facile and beneficial to the generation of sensitive and stable electrochemical signals.

Furthermore, DPV tests were performed to validate the PCR-based E-CRISPR biosensor for the detection of *S. Typhimurium*. As displayed in **Figure 4-3d**, in the absence of crRNA or target DNA (PCR products of *S. Typhimurium*), the rate of electrochemical response change (R_i) was lower than 10%, indicating the Cas12a enzyme was not activated. Only when both the target DNA and crRNA existed with Cas12a, the collateral cleavage activity of the enzyme can be activated, and R_i was significantly increased, indicating the PCR-based E-CRISPR biosensor can be used to detect *S. Typhimurium*.

4.4.3 Optimization of assay conditions

For the electrochemical sensing elements, the amount of immobilized hpDNA probes was one of the key parameters that influenced the sensitivity and stability of the overall detection. The intensity of the MB redox peak was chosen as an indicator to optimize the concentration of hpDNA. As shown in **Figure 4-4a**, when the hpDNA concentration was lower than 600 nM, it was hard to produce stable and active monolayers²⁸, resulting in the peak current fluctuating greatly. Therefore, after assessing current intensity and response efficiency, hpDNA at 800 nM was chosen for the

subsequent experiment. At this hpDNA concentration, the current signals were identical to each other, with a current change of $88.03\% \pm 1.47\%$ compared with that of the bare electrode.

Cas12a-crRNA complex, the foundation of the CRISPR/Cas12a system, dramatically influences the efficiency of collateral cleavage activity. Due to the intergroup difference of electrodes, when the electrodes are modified with the same concentration of hpDNA, the intensity of the redox peak may appear different. Besides, when the target DNA was absent on the electrode, it was observed that the redox peak had a slight reduction after E-CRISPR detection. This result can be explained by the non-specific absorption of Cas12a enzyme on the electrode and reduced the electron transfer rate. To correct these intergroup deviations, respond efficiency and R_i were used to evaluate the collateral cleavage activity (see Materials and Methods for details). Based on the previous reports²⁹, the mole ratio of Cas12a and crRNA was fixed at 1:1.5, and the Cas12a concentration was ranged from 50 nM to 250 nM. As shown in **Figure 4-4b**, the R_i increased until

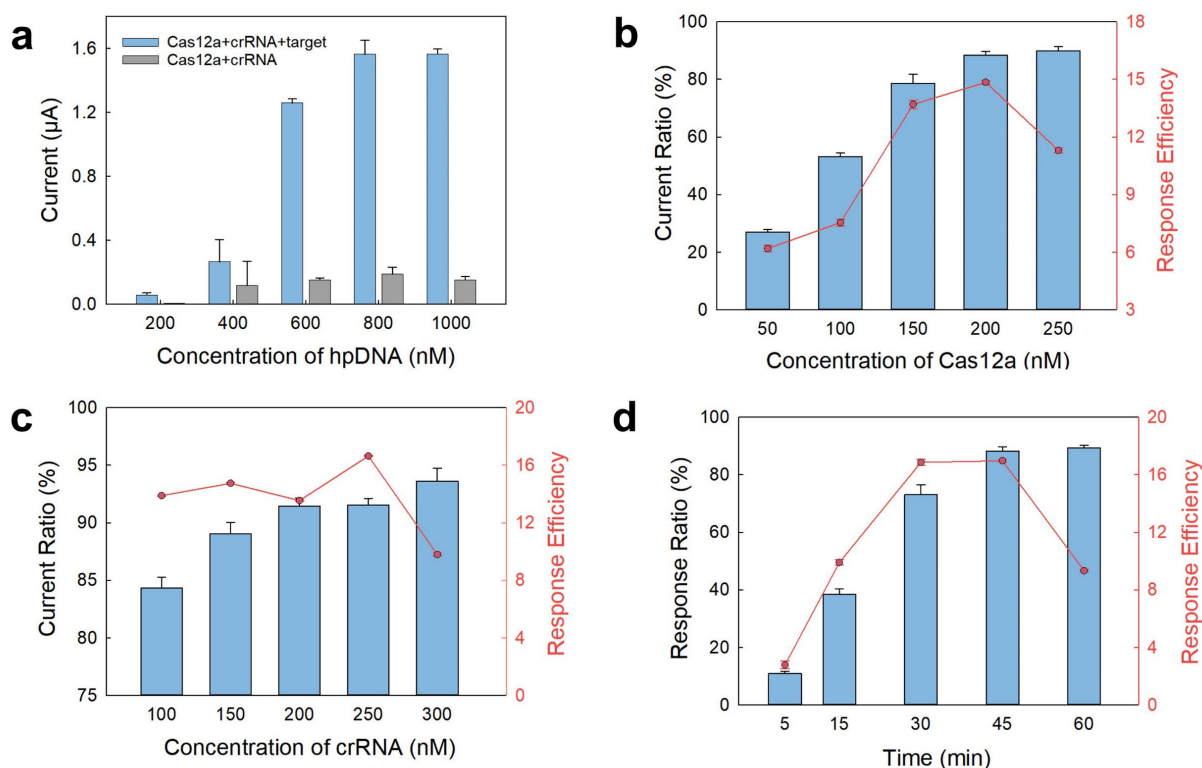


Figure 4-4 Optimization of assay parameters.

(a) The effect of hpDNA concentration on the detection signal. (b) The influence of Cas12a concentration on the electrochemical response. (c) The influence of crRNA concentration on the electrochemical response. (d) Optimization of reaction time for the collateral cleavage on the functionalized electrode. Error bars were calculated in three independent experiments.

the concentration of Cas12a exceeded 200 nM, which served as the optimum in subsequent experiments. **Figure 4-4c** shows the R_i and response efficiency at different concentrations of crRNA. As the crRNA concentration increased, the R_i and response efficiency gradually increased. When the crRNA concentration exceeded 250 nM, even though the R_i continued to increase, the background signal was also increased due to the non-specific adsorption of crRNA on the electrode surface, resulting in a decrease in response efficiency. Therefore, the concentration of crRNA was optimized to be 250 nM, in which acceptable R_i and response efficiency were obtained (**Figure 4-4c**).

The reaction time of the Cas12a-crRNA complex and target DNA on the functionalized electrode was investigated. As the reaction time increased, the R_i kept increasing until it stabilized at 45 min (**Figure 4-4d**). If the reaction time exceeds 45 min, the possibility of Cas12a and crRNA non-specific adsorb on the electrode surface may increase, leading to a reduction in response efficiency. Therefore, the optimized reaction time for the collateral cleavage was 45 min. In addition, the PCR product was cleaned up to prevent non-specific adsorption of enzymes and metal ions from the product to the electrode surface, improving the detection performance. To achieve better sensitivity, the products need to be cleaned up after the PCR procedure.

4.4.4 Analytical performance of the PCR-based E-CRISPR biosensor

For E-CRISPR analysis, the bacteria DNA was amplified by PCR first. Then, the PCR product was mixed with Cas12a-crRNA to form Cas12a-crRNA-target complex. After that, the triplex was

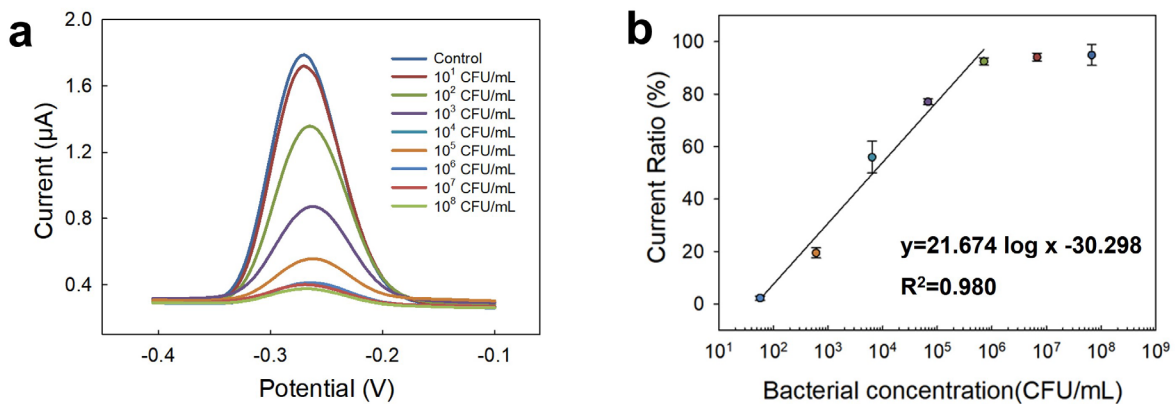


Figure 4-5 Sensitivity analysis of the proposed electrochemical sensing method.

(a) DPV curve responding for different concentrations of *S. Typhimurium* (from 6.7×10^1 CFU/mL to 6.7×10^8 CFU/mL) in PBS. (b) Plots for the detection of *S. Typhimurium* in PBS. Error bars were calculated in three independent experiments.

dropped into the functionalized electrode to trigger Cas12a collateral cleavage toward hpDNA, generating a significant change in the electrochemical signal. As seen in **Figure 4-5a**, the DPV response decreased with the increase of *S. Typhimurium* concentration. **Figure 4-5b** shows the relationship between the electrochemical responses and logarithm of *S. Typhimurium* concentration. When the *S. Typhimurium* concentration exceeded 10^6 CFU/mL, the increasing rate of R_i was slowed down, which can be explained by almost all hpDNA were digested by Cas12a. The developed biosensing method exhibits a linear detection range (LDR) from 6.7×10^1 CFU/mL to 6.7×10^5 CFU/mL, and the regression equation was described as $Y = 21.674 \log X - 30.298$ ($R^2=0.980$), where Y and X represent R_i and *S. Typhimurium* concentration, respectively. The limit of detection (LOD) was 55 CFU/mL (the electrochemical response of the control + 3 standard deviations). Compared with other reported DNA biosensors (**Table 4-1**), our proposed biosensor has a better detection limit and broader detection range. The high efficiency of PCR on DNA amplification, the high accuracy of the CRISPR system on target recognition, and the high sensitivity of electrochemical response, have cooperatively contributed to the satisfactory detection performance. Besides, according to the previous study ³, hpDNA has a lower steric hindrance effect than linear DNA, leading to the improvement of detection sensitivity.

The detection selectivity was then investigated using different competing bacteria strains, including *E. coli* O157:H7, *V. parahemolyticus*, *L. monocytogenes*, and *S. aureus* (**Figure 4-6a**). The sterilized PBS was regarded as a negative control. As shown in **Figure 4-6b**, the R_i of the competing bacteria strains (<15%) was comparable with the negative control (4.5%), whereas a

Table 4-1 Summary of the performance of DNA biosensors for the detection of *S. Typhimurium*

Method	LOD (CFU/mL)	LDR (CFU/mL)	Application
Paper chip-based device	10^2	10^2 - 10^5	Milk*
Microfluidic biosensor	10^3	-	Urine*
Optical biosensor ^{18, 19}	10^2	-	-
Label-free fluorescent aptasensor	10^2	10^3 - 10^4	-
QCM-based aptasensor	7.9×10^3	10^2 - 10^6	-
E-CRISPR biosensor	55	10^1 - 10^5	Poultry*

RPA: Recombinase Polymerase Amplification LAMP: Loop-Mediated Isothermal Amplification; QCM: Quartz Crystal Microbalance; * represents the method that needs sample preparation.

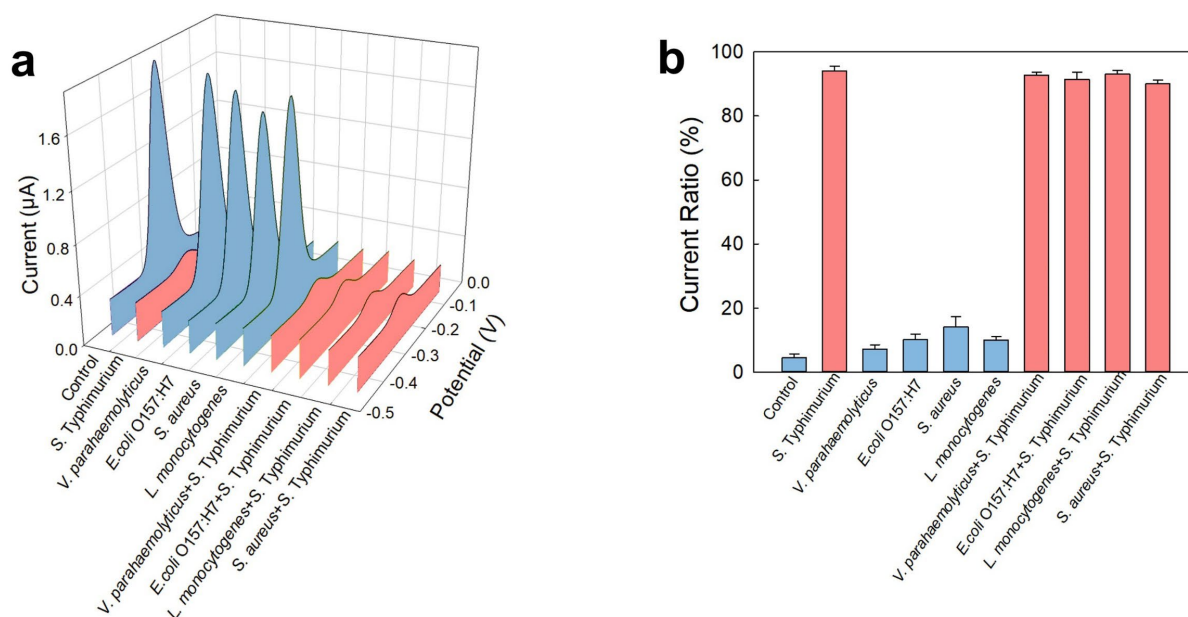


Figure 4-6 The selectivity of the biosensor.

(a) DPV curve responding to different bacteria. (b) Comparison of the electrochemical signal response obtained for different bacteria. The concentrations of *S. Typhimurium* and the competing bacteria are 1×10^7 CFU/mL. Error bars were calculated in three independent experiments. Statistical significance was shown as follows: $**P < 0.01$, target signal vs. control.

significant increase in R_i was observed for *S. Typhimurium* ($>90\%$). Moreover, the anti-interference capability of this biosensor was evaluated by mixing *S. Typhimurium* with the competing bacteria strains. The R_i was increased to the same level as pure *S. Typhimurium*. The high selectivity and anti-interference capability toward *S. Typhimurium* detection can be explained for the following two reasons. First, the PCR primers were designed to perfectly match the target DNA sequence, conferring exquisite specificity and enabling the E-CRISPR system to readily distinguish bacteria-of-interest from the non-target DNA. Second, due to the extremely high affinity and specificity of the Cas12a-crRNA complex, the CRISPR system could accurately identify and cleave the target DNA.

4.4.5 Detection performance for *S. Typhimurium* in poultry meat

To validate the performance of the proposed E-CRISPR biosensor with food samples, the poultry meat spiked with various concentrations of *S. Typhimurium* was analyzed by our proposed PCR-based E-CRISPR method. As depicted in **Figure 4-7a**, the DPV signals exhibited a gradual decrease from 3.3×10^2 CFU/mL to 3.3×10^6 CFU/mL. A calibration curve was established (**Figure**

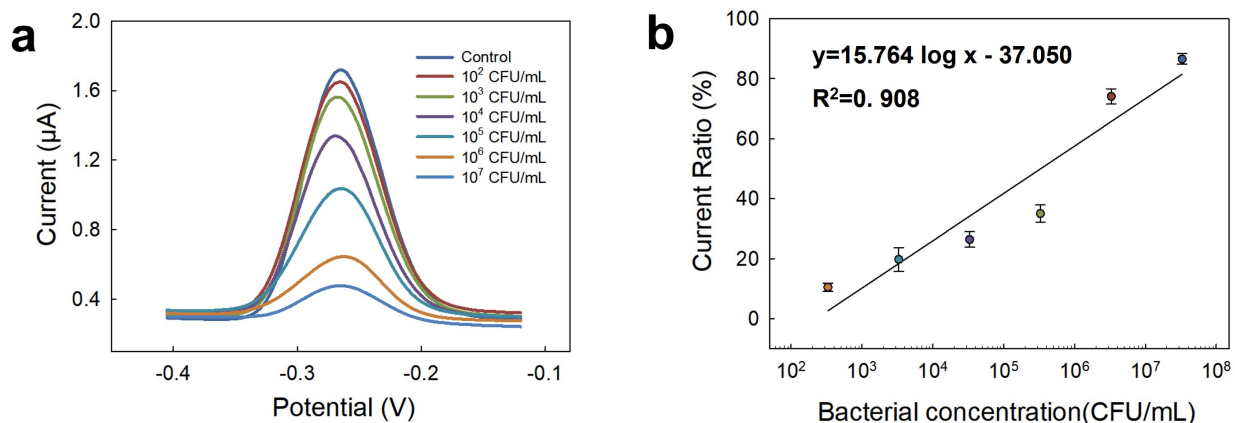


Figure 4-7 Detection of *S. Typhimurium* in spiked poultry meat.

(a) DPV curves respond to various concentrations of *S. Typhimurium* (from 3.3×10^2 CFU/mL to 3.3×10^7 CFU/mL) in spiked poultry meat. (b) Calibration curve for the detection of *S. Typhimurium* in spiked chicken meat. Error bars were calculated in three independent experiments.

4-7b); the regression equation is $y = 15.764 \log x - 37.050$ ($R^2 = 0.908$), where y and x represent R_i and *S. Typhimurium* concentration, respectively. The LOD in the poultry sample was 820 CFU/mL ($S/N=3$), approximately one order of magnitude higher than the LOD in pure culture. This result might be caused by the food components (such as fat, protein and carbohydrate) in the complex matrix of poultry meat. The non-specific adsorption of food components on the electrode surface could bind with the hpDNA, resulting in higher background noise and lower collateral cleavage efficiency. Interestingly, after comparing with other DNA biosensor applications in **Table 4-1**, only the proposed biosensor was used in the solid sample, which might be because the sample pretreatment of solid food sample is much more challenging than the liquid sample (such as milk and urine). In addition, compared with the liquid sample, the matrix in the solid sample will be more complex due to the higher concentration of proteins and lipids, especially for the meat sample, which requires higher robustness of the detection method.

4.5 Conclusion

In this study, an E-CRISPR electrochemical sensing method was developed for the rapid and sensitive detection of *S. Typhimurium*. Converging the advantages of PCR amplification and E-CRISPR, the integrated biosensor significantly promoted the detection sensitivity and specificity. In the optimized conditions, the PCR-based E-CRISPR biosensor has LODs of 55 CFU/mL in PBS. Benefiting from the strong anti-interference ability and robustness, this biosensor can also be

applied in poultry meat with LODs of 820 CFU/mL. The total detection time for *S. Typhimurium* in the chicken sample was less than 2.5 h. Compared with the traditional microbiological methods, this E-CRISPR biosensor shows advantages of rapidity, simplicity, sensitivity, and specificity. So far, although PCR is regarded as the gold standard for molecular diagnostics, it still has much room for improvement. The introduction of E-CRISPR can remedy the shortcomings of PCR. As a tool for further detection, it is able to prevent false positive results due to PCR amplification of unspecific genes, which significantly facilitates the accuracy of the detection.

In the future, taking advantage of the programmability of crRNA, the PCR-based E-CRISPR biosensor can be easily extended to different application scenarios, such as the detection of single nucleotide polymorphism in the pathogen. Besides, to apply E-CRISPR biosensor in resource-poor areas, PCR can be replaced by isothermal DNA amplification technology (such as RPA, LAMP) that does not require special instruments, providing a promising detection platform in the food supply chain.

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Chapter 5

Enhancing CRISPR/Cas12-based biosensors sensitivity via site-specific Cas12-crRNA crosslinking

5.1 Abstract

CRISPR/Cas12a-based diagnostics have emerged as powerful tools for nucleic acid detection due to their unique trans-cleavage activity. However, the dynamic and relatively weak interaction between Cas12a and its CRISPR RNA can limit sensitivity. In this study, we developed a site-specific crosslinking strategy to enhance the detection performance of the CRISPR/Cas12a system. By genetically incorporating the latent bioreactive unnatural amino acid fluorosulfate-L-tyrosine (FSY) into Cas12a, we achieved covalent conjugation with crRNA through a proximity-enabled sulfur-fluoride exchange reaction. Four FSY-modified variants were evaluated, all showing improved trans-cleavage activity compared to the wild-type enzyme. Among them, AsCas12a-H800FSY was selected for further study due to its flexible structure. Furthermore, the FSY-modified CRISPR system was integrated with reverse transcription and isothermal amplification, resulting in enhanced sensitivity for SARS-CoV-2 detection, with a limit of detection as low as 10^1 copies/ μ L. This work provides a robust and modular platform for enhancing CRISPR-based diagnostics, with broad potential for point-of-care detection of viral pathogens.

Keywords: CRISPR, unnatural amino acid, biosensor, SARS-CoV-2

5.2 Introduction

The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) systems, originally discovered as bacterial adaptive immune systems against phage infections, have emerged as versatile tools in gene editing and molecular diagnostics.¹⁻⁴ At the core of these systems are CRISPR-associated (Cas) proteins and their corresponding CRISPR RNAs (crRNAs), which enable precise, sequence-specific target recognition and cleavage. For instance, the Cas9 enzyme employs a two-part guide RNA system—comprising a crRNA and a tracrRNA fused into a single-guide RNA (sgRNA) of approximately 100 base pairs—to mediate precise DNA recognition and cleavage exclusively via cis-cleavage activity.⁵ In contrast, the Cas12 enzyme utilizes a more

compact crRNA of approximately 42 base pairs and exhibits both cis- and trans-cleavage activities, the latter enabling signal amplification and expanding its utility in molecular diagnostics.⁶ However, the interaction between Cas enzyme and crRNA is generally dynamic, transient and weak. Previous studies have shown that the dissociation constant of the Cas12-crRNA complex typically falls within the nanomolar range,^{7, 8} which is higher than the picomolar range observed for the Cas9-sgRNA complex.⁹ This discrepancy suggests that the shorter length of the Cas12 crRNA may contribute to its comparatively lower binding efficiency, potentially influencing its sensitivity in diagnostic applications.

To improve the sensitivity of CRISPR/Cas12 system, some research enhanced the interaction between Cas12 and crRNA.^{10, 11} Liu's group synthesis the 5' terminal modified crRNA with dibenzocyclooctyne (DBCO) and the site-specifically modified Cas12a with azido group. Through a strain-promoted alkyne-azide cycloaddition reaction, the crRNA was anchored on the Cas12 enzyme.¹⁰ As a result, the Cas12-crRNA complex displayed a higher efficiency in genome knockin than that of the wild-type system. However, the modification on both crRNA and Cas enzyme increases cost and complexity of experiments. Furthermore, this strategy has been mainly applied in gene editing, leaving substantial potential for exploration in diagnostic applications.

Herein, we proposed a universal method for Cas12 enzyme-crRNA conjugation to enhance the sensitivity of CRISPR system. The latent bioreactive unnatural amino acid, fluorosulfate-l-tyrosine (FSY),^{12, 13} was site-specific modified on the Cas12 enzyme. In the presence of crRNA, the FSY-modified Cas12 enzyme is able to react with crRNA through proximity-enabled sulfur-fluoride exchange (SuFEx) reaction,¹⁴ forming a covalent bond between FSY-modified Cas12 enzyme and crRNA. The cis-cleavage and trans-cleavage of crosslinked CRISPR systems were characterized separately. Leveraging trans-cleavage ability, a CRISPR/Cas12-based biosensor was constructed for the detection of SARS-CoV-2. Compared with the wild-type CRISPR system, the crosslinked CRISPR system achieved a higher trans-cleavage activity and a more sensitive performance in the detection. To the best of our knowledge, this is the first study to utilize latent bioreactive unnatural amino acid for improving the sensitivity of CRISPR-based biosensor. Without the requirement of RNA modification, the proposed CRISPR system can be easily adapted to different diagnosis scenarios by simply altering the crRNA. This innovative approach paves the way for more accessible and versatile CRISPR diagnostics.

5.3 Materials and Methods

5.3.1 Materials

Escherichia coli DH10 β (*E. coli* DH10 β) was purchased from Fisher Scientific (Hampton, NH). The imidazole, LB broth, agar, ampicillin and chloramphenicol were purchased from Research Products International (Mount Prospect, IL). The PCR master mix, M13 circular ssDNA, DNA loading dye, DNase I, DNA cleanup kit, PCR cloning kit, and plasmid extraction kit were purchased from New England Biolabs (Ipswich, WA). The DNA oligoes, primers and crRNA are synthesized by Integrated DNA Technologies (Coralville, IA). The SARS-CoV-2 E gene DNA was synthesized by Twist Bioscience (San Francisco, CA). The inactive virus samples were requested from Biodefense and Emerging Infections Research Resources Repository (BEI resources, Manassas, VA). The fluorosulfate-L-tyrosine (FSY) was kindly provided by Dr. Wei Sun's group at Virginia Tech. The RPA kit was purchased from TwistDx (Maidenhead, UK). All other chemicals were purchased from Fisher Scientific (Hampton, NH).

5.3.2 Plasmid construction

Two plasmids were utilized for protein expression: pBAD-AsCas12a plasmid, containing the AsCas12a coding sequence with an amber mutation, and pEvol-FSYRS plasmid, which encodes an FSY-specific synthetase and tRNA^{Py1} for expression in *E. coli* DH10 β cells.

To construct pBAD-AsCas12a-WT plasmid, the DNA sequences encoding AsCas12 protein were cloned into a pBAD-based expression vector containing a C-terminal 10 $\times$ His-tag. Site-directed mutagenesis was employed to generate four different pBAD-AsCas12a-TAG plasmids from the pBAD-AsCas12a-WT plasmid. In these constructs, codons corresponding to residues 746, 754, 800, 806, and 856 of the AsCas12a protein were individually mutated to the stop codon TAG. Besides, the pEvol-FSYRS plasmid was a gift from Dr. Wei Sun's group at Virginia Tech.

5.3.3 Protein expression and purification

The pBAD-AsCas12a plasmid and pEvol-FSYRS plasmid were co-transformed into *E. coli* DH10 β chemical competent cells for protein expression. A single colony of *E. coli* DH10 β containing both plasmids was inoculated into LB medium supplemented with 100 μ g/mL ampicillin and 34 μ g/mL chloramphenicol, followed by incubation at 37°C overnight with agitation. The following day, 0.5 mL of the overnight culture was transferred into 50 mL of 2 $\times$ YT

broth containing 100 µg/mL ampicillin and 34 µg/mL chloramphenicol. The culture was grown at 37°C with agitation until the optical density at 600 nm (OD₆₀₀) reached 0.4–0.6. Protein expression was induced by adding 0.2% arabinose and 1 mM FSY, and the culture was incubated at 18°C for 18 hours. Cells were harvested by centrifugation at 4,200×g for 30 minutes at 4°C. The cell pellets were stored at –80°C.

5.3.4 Cas12a-crRNA crosslinking assay

The Cas12a-crRNA crosslinking assays were performed with 100 nM AsCas12a protein and 50 nM crRNA in a reaction buffer containing 10 mM Tris-HCl, 50 mM NaCl, and 10 mM MgCl₂. The AsCas12a/crRNA mixtures were incubated at 37°C overnight. After that, the samples were denatured with the Laemmli sample buffer at 95°C for 5 min. The denatured products were characterized through denaturing gel electrophoresis on 10% 8M urea-TBE-PAGE. The gel images were captured by iBright Imaging Systems (Thermo Fisher Scientific, Waltham, MA).

5.3.5 Characterization of *cis*- and *trans*-cleavage activity

A 1500bp dsDNA fragment was prepared for *cis*-cleavage activity experiment. The dsDNA was ligated into pMiniT 2.0 vector through NEB PCR cloning kit. The ligation product was transformed into *E. coli* DH10β chemical competent cell. The insert sequence in the plasmid was confirmed by Sanger sequencing. The 1500 bp dsDNA fragment was subsequently amplified from the plasmid using PCR and purified with a DNA Cleanup Kit. To initialize the *cis*-cleavage of CRISPR system, the dsDNA target was added into the Cas12/crRNA mixture, which consisted of 100 nM AsCas12a, 60 nM crRNA, and 1x NEBuffer 2.1. The reaction was carried out at 37°C for 30 min. The reaction products were analyzed by 1.5% agarose gel.

For *trans*-cleavage activity test, the 100nM AsCas12a was incubated with 60 nM crRNA and 10nM ssDNA in 1x NEBuffer 2.1 at 37°C for 30 min. After that, the M13mp17 ssDNA was added into the CRISPR reaction and incubated at 37°C for indicated timepoints. Reactions were quenched with DNA loading dye and then characterized by 1% agarose gel.

5.3.6 CRISPR biosensor for the detection of SARS-CoV-2 RNA

The Cas12a reaction consisted of 100 nM AsCas12a, 50 nM crRNA, 50 nM ssDNA-FQ probe, and 1×NEBuffer2.1 in a total reaction volume of 50 µL. The fluorescence signal (λ_{ex} :485 nm, λ_{em} :528 nm) of the reaction was measured every 1 min with a BioTek Synergy H1 Plate Reader

(Agilent Technologies, Santa Clara, CA). The target DNA and ssDNA-FQ probe were listed in **Table 5-1**.

5.3.7 Reverse transcription recombinase polymerase amplification (RT-RPA)

RT-RPA reactions were carried out with TwistAmp Basic (TwistDx) with the addition of M-MuLV Reverse Transcriptase and Murine RNase Inhibitor. The RT-RPA reactions were conducted at 42°C for 45 min at the T100 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). The total volume of each reaction is 50 µL, which contains 29.5 µL of rehydration buffer, 2.4 µL of forward primer (10 µM), 2.4 µL of reverse primer (10 µM), 1 µL of transcriptase (200 U/µL), 1 µL of RNase inhibitor (40 U/µL), 2.5 µL of magnesium acetate (280 mM), 10.2 µL of water and 1 µL template.

5.3.8 Michaelis-Menten analysis

The Michaelis-Menten analysis was used to evaluate the trans-cleavage activities of WT AsCas12a and mutant AsCas12a on the SARS-CoV-2 E gene (dsDNA). The 100 nM crRNA was premixed with 200 nM AsCas12a and incubated at 37°C for 30 min. Subsequently, 10 nM target dsDNA was added into the Cas12a/crRNA complex and then incubated at 37°C for 30 min. Active AsCas12a ternary complexes were considered to have a final concentration of 10 nM.

The reaction was initiated by incubating 1 nM active AsCas12a ternary complexes with different concentrations ssDNA reporters (15.63, 31.25, 62.5, 125, 250, 500, 1000, 2000 nM) at

Table 5-1 The sequence of primers and crRNA

Name	Sequence (5' to 3')
Forward primer	cggaagagacaggtacgttaatagttaatagc
Reverse primer	agaccagaagatcaggaactctagaagaat
E gene	cggaagagacaggtacgttaatagttaatagcgtactcttttcttgcttctggtattcttgctagttacact agccatccttactgcgcttcgattgtgtgcgtactgctgcaatattgtaacgtgagtcctgtaaaccttctttt tacgtttactctcgtgttaaaaatctgaattcttctagagttcctgatcttctggtctaa
crRNA	UAAUUUCUACUCUUGUAGAUGUGGUAUUCUUGCUAGUUACA

37°C for 30 min. The fluorescence signal of the reaction was measured every 1 min with a BioTek Synergy H1 Plate Reader (Agilent Technologies, Santa Clara, CA).

A fluorescence calibration curve was used to convert relative fluorescence units to molar concentration (in nM). To avoid the bias caused by different Cas enzymes, DNase I (100 U) was used to construct the fluorescence calibration curve. The details were followed by the previous report.¹⁵ The initial velocity was calculated by fitting a linear regression and plotted against the substrate concentration in the first 15 min. The reaction velocity versus substrate concentration data were fitted to the Michaelis-Menten equation using GraphPad Prism 9 to calculate kinetic parameters K_{cat} and K_M .

5.3.9 Clinical Samples and ethics statement

The SARS-CoV-2 clinical samples were obtained from Children's National Hospital (Washington, D.C.) under their ethical approvals. RT-qPCR was performed through Luna® SARS-CoV-2 RT-qPCR Multiplex Assay Kit (NEB, Ipswich, MA).

5.4 Results and Discussion

5.4.1 Characterization of mutant AsCas12a

To incorporate the FSY into Cas12a nuclease, the genetic code expansion was involved in the protein expression process (**Figure 5-1a**). The key mechanism of genetic code expansion was recording an amber mutation (TAG) to code for unnatural amino acid (FSY) through using an orthogonal pair of pyrrolysyl tRNA ($tRNA^{Pyl}$) and FSY-specific synthetase (FSYRS). With the help of FSY-specific synthetase, the FSY was attached to the $tRNA^{Pyl}$, and then incorporated at the designated codon position within the Cas12a sequence. The FSY targeted the nucleophilic group of RNA, which could be a 2'-hydroxy group of ribose or the amino groups of on a base. This proximity-enabled SuFEx reaction generated the covalent binding between FSY-modified Cas12a and crRNA, opening up multiple possibilities for the further application.

After inspecting the crystal structure of AsCas12a/crRNA/target DNA ternary complex (**Figure 5-1b**), five residues of AsCas12a were chosen as candidates for FSY incorporation separately, which are Y746, H754, H800, M806, and H856. These residues are close to crRNA and most of them have similar structures to FSY. The wild-type AsCas12a (AsCas12a-WT), AsCas12a-Y746FSY, AsCas12a-H754FSY, AsCas12a-H800FSY, AsCas12a-M806FSY, and AsCas12a-H856FSY were expressed in *E. coli* DH10 β . The cell lysates were analyzed by western blot

(Figure 5-1c). The results showed that four out of five mutant AsCas12a were expressed successfully in the presence of FSY. The conjugation between mutant AsCas12a and crRNA was confirmed by denaturing urea-PAGE (Figure 5-1d). Clear conjugation bands were observed for all purified mutants, whereas no crosslinking was observed between wild-type AsCas12a and crRNA.

5.4.2 Cleavage ability of AsCas12a nucleases

Cis-cleavage and trans-cleavage are two critical abilities of Cas nucleases. Cis cleavage is target dependent, while trans-cleavage is non-specific. In the presence of target sequence, the AsCas12a is activated by the hybridization of guide RNA and target DNA, triggering the cis-

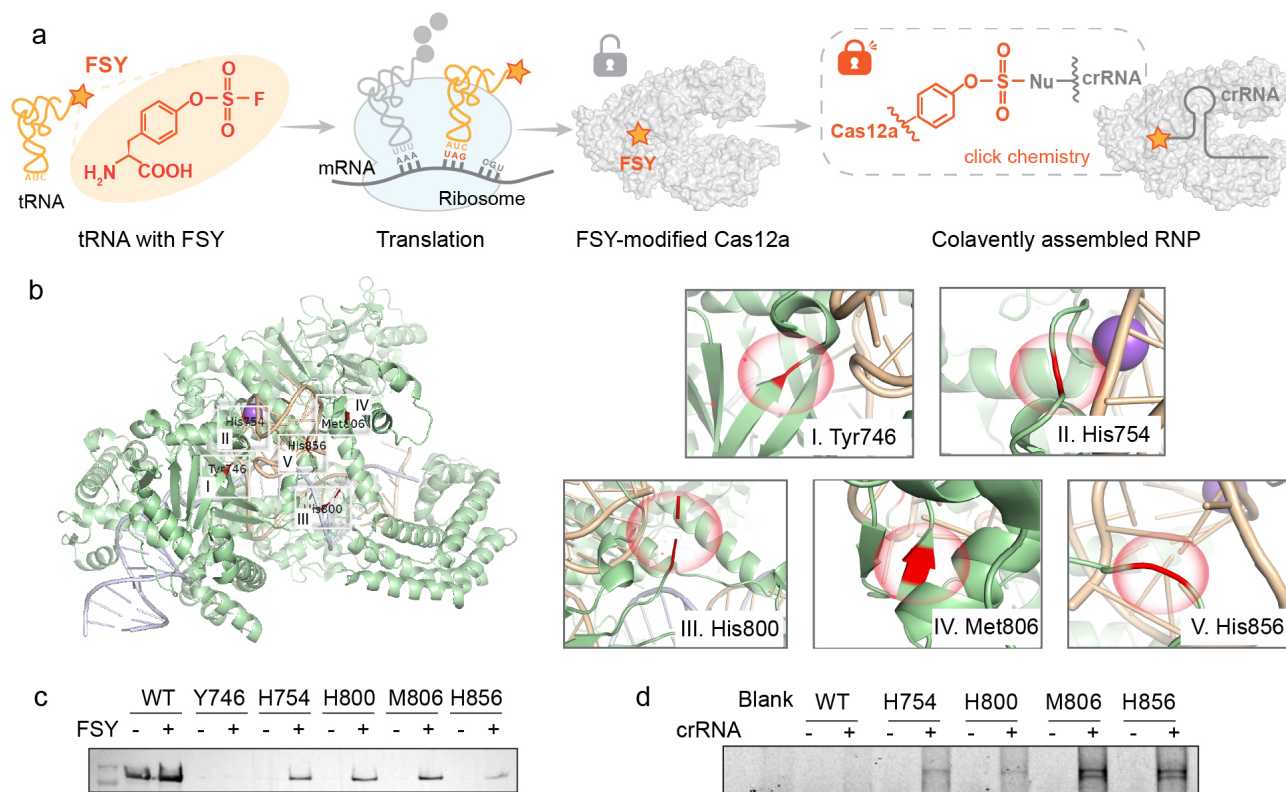


Figure 5-1 Site-specific crosslinking of AsCas12a nuclease with crRNA.

Scheme of AsCas12a nuclease and crRNA crosslinking. AsCas12a was site-specific modified with FSY. In the presence of crRNA, the FSY attacked the nucleophilic group of crRNA, generating the covalent binding between AsCas12a nuclease and crRNA. (b) Crystal structure of Cas12a/crRNA/target DNA ternary complex (PDB: 5b43). The residues selected for FSY mutagenesis are labeled in red. (c) Western blot analysis of the bacterial lysates. The bacteria were treated with or without FSY during protein. (d) Characterization of Cas12 nuclease-crRNA conjugation using denaturing urea-PAGE.

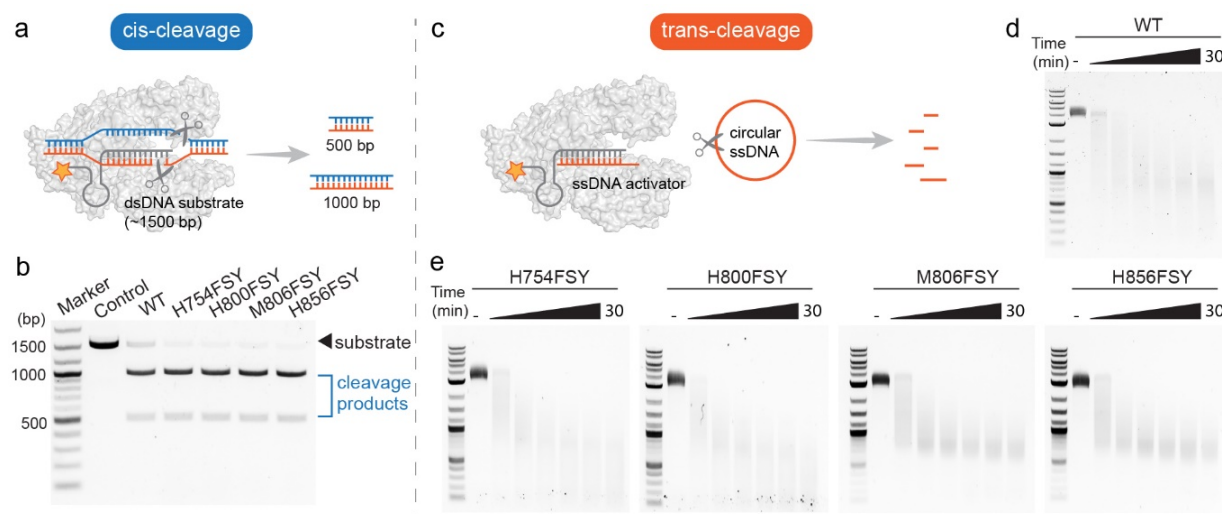


Figure 5-2 Characterization of cleavage activities.

Characterization of cis-cleavage and trans-cleavage of WT AsCas12a and four mutants (H754FSY, H800FSY, M806FSY, and H856FSY). (a) Scheme of cis-cleavage of AsCas12a/crRNA toward dsDNA substrate (1500bp). (b) The dsDNA substrate was digested in the presence of AsCas12a/crRNA complex for 15 min at 37°C. (c) Scheme of trans-cleavage of AsCas12a/crRNA toward circular ssDNA substrate (M13 phage plasmid). (d-e) The ssDNA substrates were digested by WT (d) and mutant (e) AsCas12a/crRNA complex in 30 min.

cleavage of the target and trans-cleavage of the nontarget ssDNA nearby. To evaluate cis-cleavage of CRISPR/AsCas12a systems, a ~1500bp dsDNA was designed as the target sequence. With the guide of crRNA, the AsCas12a systems were able to recognize the ~1500 bp target sequence and active the cis-cleavage (**Figure 5-2a**). As a result, the target sequence would be digested into ~500 bp and ~1000 bp fragments. The cleaved fragments were characterized through gel electrophoresis (**Figure 5-2b**). The results showed that WT and all mutant AsCas12a have cis-cleavage activity. The circular ssDNA was employed on trans-cleavage verification (**Figure 5-2c**). After the AsCas12a nucleases activated by ssDNA target, circular ssDNA was added into the CRISPR reaction. The reaction products were collected every 5 min. DNA gel results demonstrated that the WT and mutant AsCas12a could digest the ssDNA in 15 min, proving that the trans-cleavage ability of each AsCas12a nucleases (**Figure 5-2 d and 5-2e**).

5.4.3 CRISPR/AsCas12a-based biosensor for SARS-CoV-2 detection

Encouraged from the previous results, a CRISPR/AsCas12a-based biosensor was designed for the detection of SARS-CoV-2 (**Figure 5-3a**). The crRNA was designed to target the small envelope protein gene (E gene), which is a highly conserved region among coronaviruses.¹⁶ Mutant

AsCas12a was premixed with crRNA to facilitate anchoring of the crRNA to AsCas12a via a proximity-enabled SuFEx reaction. In the presence of E gene dsDNA, the AsCas12a/crRNA was recognizing the target and triggering the cleavage activity. The fluorophore-quencher probe (FQ-probe) was designed to generate a fluorescent signal during trans-cleavage. It consists of single-stranded DNA (ssDNA) with a fluorophore and quencher attached at opposite ends.

The trans-cleavage activity was quantified using Michaelis-Menten kinetics (**Figure 5-3b**). The E gene dsDNA served as the activator to initiate trans-cleavage, while varying concentrations of the FQ-probe were used as substrates. Initial reaction velocities were fitted to the Michaelis-Menten model to calculate the kinetic parameters K_{cat} and K_M (**Figure 5-3c**). The ratio between K_{cat} and K_M is reflected in the enzymatic activity of the corresponding AsCas12a. The results showed that all mutants have a higher enzymatic activity of trans-cleavage than that of WT, proving that the crosslinking between AsCas12a and crRNA increase the trans-cleavage activity. However, there is no significant difference among mutants in terms of K_{cat}/K_M . We speculate that all mutants have similar trans-cleavage activity, which is due to cross-linking and otherwise does not affect activity. Due to the H800 residues located in the unsolved crystal structure of AsCas12a, it gave the H800 residue much more flexible programmability than other selected residues. Therefore, the AsCas12a-H800FSY was chosen for the further experiments.

To evaluate the performance of AsCas12a-H800FSY in detection, various concentrations of the SARS-CoV-2 E gene (dsDNA) were tested using the CRISPR/AsCas12a-H800FSY system. The detection sensitivity of the mutant system was compared with that of the WT system. In the WT system, fluorescence intensity increased with higher concentrations of the SARS-CoV-2 E gene (**Figure 5-3d**). A linear relationship between fluorescence intensity and target DNA concentration was observed from 0.01 nM to 0.5 nM (**Figure 5-3e**), with a regression equation of $y = 14,525x + 466$, where y is fluorescence intensity (a.u.) and x is target concentration (nM). The limit of detection (LOD) was calculated to be 16.1 pM ($S/N = 3$). Similarly, the mutant system was used to detect varying concentrations of DNA targets (**Figure 5-3f**), showing a regression equation of $y = 21,969x + 649$. Compared to the WT system, the mutant system achieved higher sensitivity, with an LOD as low as 9.51 pM. This enhanced performance may be attributed to the covalent

bond between AsCas12a-H800FSY and crRNA, which improves the trans-cleavage efficiency of the CRISPR system.

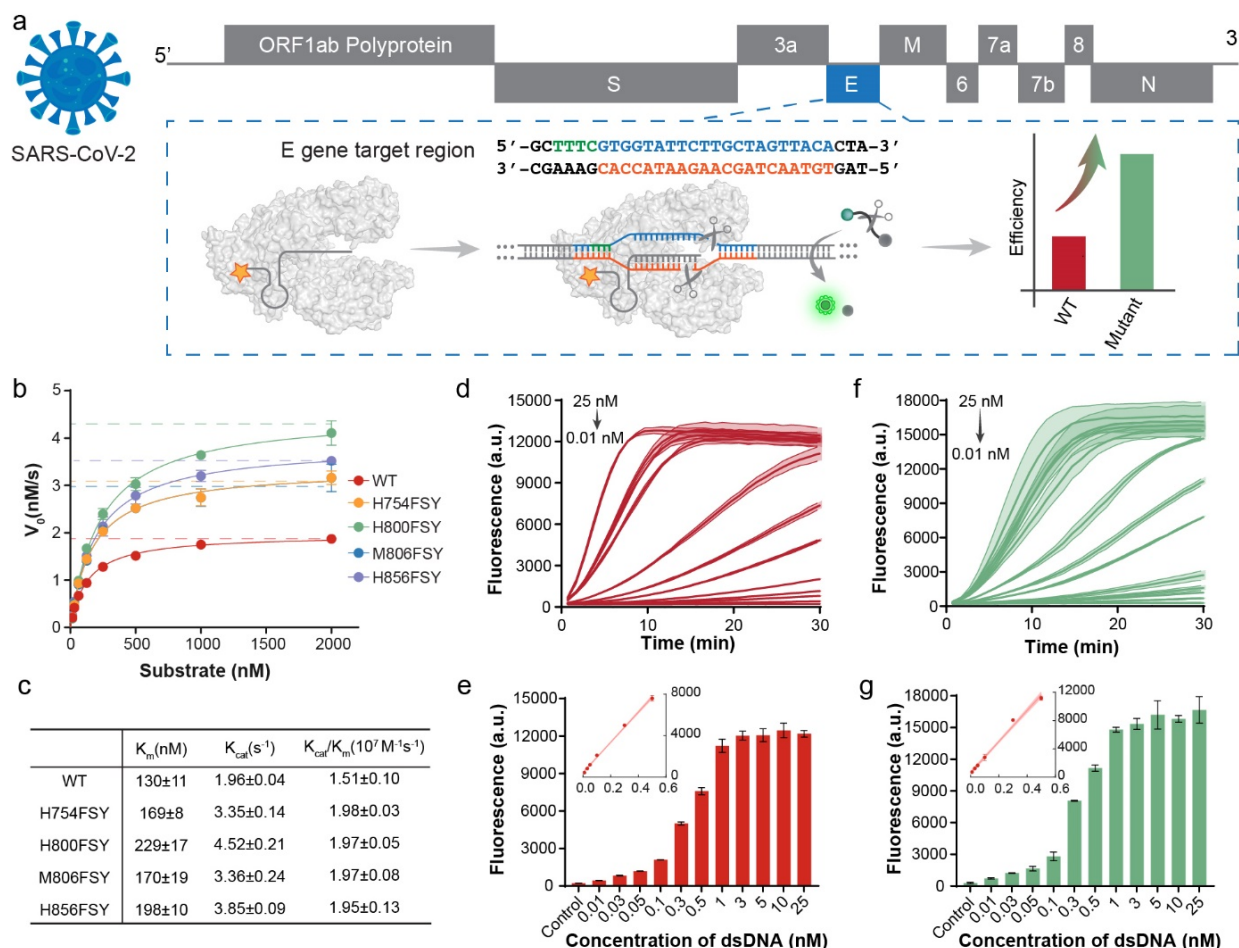


Figure 5-3 The AsCas12a-based biosensor for the detection of SARS-CoV-2.

(a) Schematic illustration of SARS-CoV-2 detection. The crRNA was designed to target the E gene of SARS-CoV-2. In the presence of target DNA, the AsCas12a/crRNA system recognized and cleaved the target, generating the green fluorescent as the signal output. (b-c) Michaelis-Menten analysis of different AsCas12a enzyme with dsDNA activator (E gene). The H800FSY showed the highest enzyme activity. (d, e) Real-time fluorescence kinetics (d) and fluoresce response (e) of the AsCas12a-WT/crRNA system with different concentration target dsDNA. (f, g) Real-time fluorescence kinetics (f) and fluorescent response (g) of the AsCas12a-H800FSY/crRNA system with different concentration target dsDNA.

5.4.4 Real sample application

After evaluating the detection performance for the E gene DNA, the proposed biosensor was integrated with isothermal amplification to improve its applicability and sensitivity for viral RNA detection. Given that SARS-CoV-2 is a positive-sense single-stranded RNA virus, reverse

transcription recombinase polymerase amplification (RT-RPA) was employed to amplify the E gene directly from virus lysates. The resulting amplicons were then analyzed using the AsCas12a-H800FSY/crRNA complex. Upon activation by the target sequence, the FSY-modified Cas12a cleaved the FQ-probe, generating a green fluorescence signal (**Figure 5-4a**).

The detection performance of both the wild-type (WT) and mutant AsCas12a systems was further evaluated using viral RNA samples. Six different viruses—H1N1, H7N2, MERS-CoV, Influenza B, HCoV-NL63, and SARS-CoV-2—were obtained from BEI Resources. After lysis at 70 °C for 5 minutes, the viral lysates were subjected to detection using the proposed CRISPR-

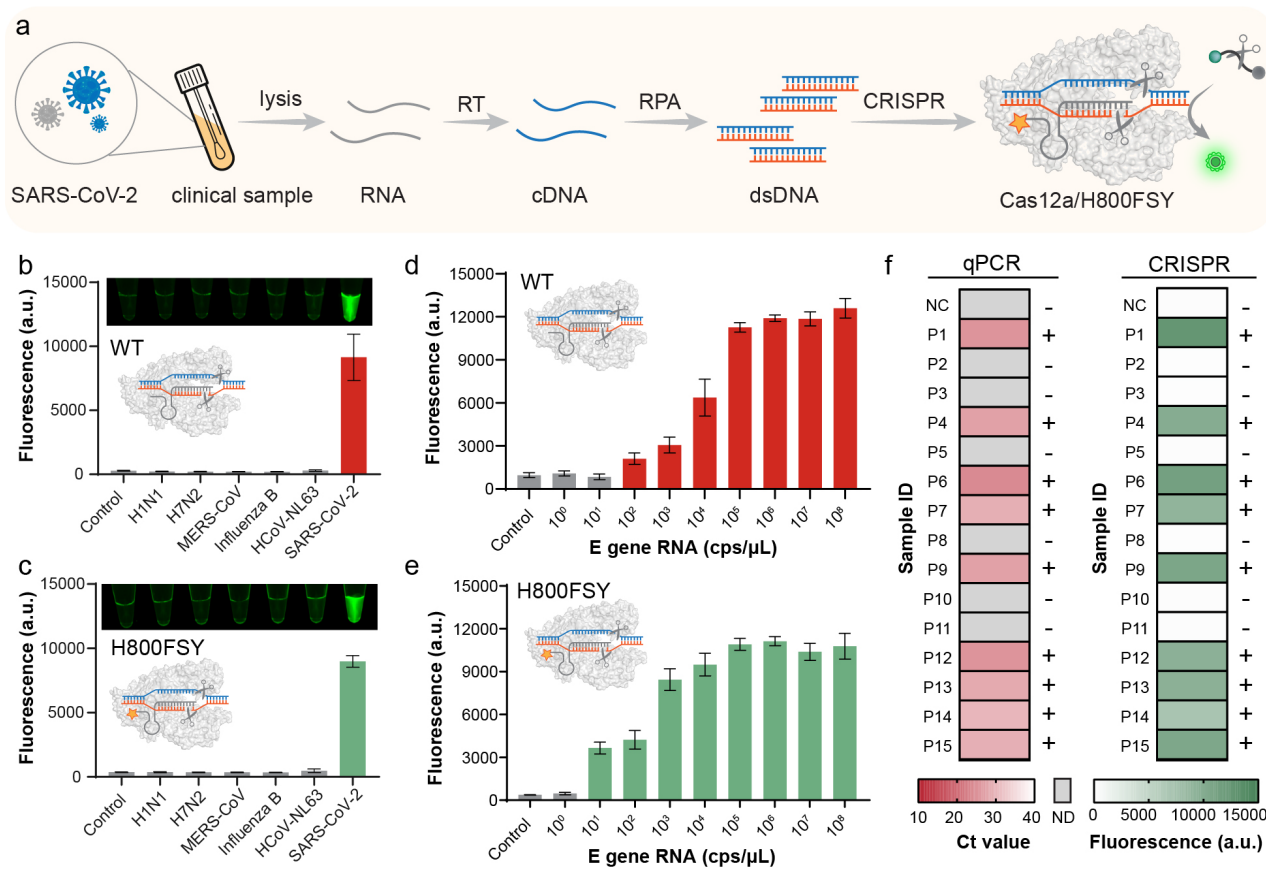


Figure 5-4 The clinical application of AsCas12a-based biosensor.

(a) Workflow for SARS-CoV-2 detection. Viral RNA was released from clinical samples and amplified using RT-RPA at 42°C. The AsCas12a /crRNA system recognized the target and generate fluorescent. (b, c) The selectivity test of WT (b) and H800FSY (c) systems against six different viruses. (d, e) Sensitivity comparison of WT (d) and H800FSY (e) systems for detecting SARS-CoV-2 RNA. (f) Detection results of SARS-CoV-2 from 15 clinical samples using qPCR and CRISPR/AsCas12a-H800FSY. NC means negative control. ND means not detected.

based biosensor. Fluorescent images of each reaction were captured using a fluorescence imager, and the corresponding fluorescence intensities were recorded. As shown in **Figures 5-4b and 5-4c**, both the WT and H800FSY mutant systems accurately distinguished SARS-CoV-2 from the other five viruses, demonstrating high specificity. In addition, both systems were tested across a range of SARS-CoV-2 E gene RNA concentrations. The WT system achieved a detection limit of 10^2 copies/ μ L (**Figure 5-4d**), whereas the H800FSY mutant system reached a limit of 10^1 copies/ μ L (**Figure 5-4e**), indicating a ten-fold improvement in sensitivity. These results confirm the enhanced performance of the FSY-modified CRISPR system for sensitive and specific detection of viral RNA.

As a practical application, the proposed biosensor was used to detect SARS-CoV-2 in clinical samples. A total of 15 nasopharyngeal swab samples were collected from Children's National Hospital. To release viral RNA, the samples were incubated at 95 °C for 5 minutes. The lysates were then added to RT-RPA reaction mixtures to initiate reverse transcription and DNA amplification. The resulting products were analyzed using the CRISPR/AsCas12a-H800FSY detection system. The entire process was completed within 1.5 hours. Among the 15 samples, 9 produced strong fluorescence signals following the CRISPR reaction (**Figure 5f**). The results were consistent with those obtained from a commercial RT-qPCR kit, confirming that the proposed method offers comparable accuracy while reducing assay time and complexity.

5.5 Conclusion

In summary, we developed a site-specific crosslinking strategy to enhance the detection sensitivity of the CRISPR/Cas12a system. By incorporating the latent bioreactive unnatural amino acid (FSY) at a designated site on Cas12a, covalent conjugation with CRISPR RNA was achieved, resulting in a more stable and robust Cas12a–CRISPR RNA complex. This modification significantly improved the trans-cleavage activity and detection sensitivity of the biosensor without requiring chemical modification of the RNA. The FSY-modified CRISPR system was successfully applied to detect SARS-CoV-2 with high sensitivity and specificity when combined with isothermal amplification. This work presents a universal and adaptable approach for improving CRISPR-based diagnostics, offering strong potential for point-of-care applications and the detection of emerging pathogens.

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Chapter 6

Simultaneous dual-gene detection of *Escherichia coli* O157:H7 based on CRISPR/Cas13-mediated biosensor

6.1 Abstract

Escherichia coli O157:H7 is a major foodborne pathogen that poses significant public health risks, requiring rapid and accurate detection methods. In this study, we developed a one-pot CRISPR/Cas13-based biosensor for the simultaneous detection of two key genetic markers, *rfbE*_{O157} and *fliC*_{H7}. By leveraging the distinct trans-cleavage preferences of LwaCas13a and PsmCas13b, the biosensor establishes dual fluorescence channels (TEX and FAM), enabling serotype-specific detection while minimizing false positives. The entire detection process (target gene amplification, transcription, and CRISPR-mediated biosensing) was integrated into a single reaction tube, eliminating the need for complex equipment and reducing the risk of contamination. The biosensor demonstrated high sensitivity, achieving a detection limit of 54 CFU/mL using recombinase polymerase amplification (RPA). To validate its real-world applicability, an *E. coli* O157:H7 infection model was established in mice, and fecal samples were analyzed, showing results consistent with standard PCR method. Compared to conventional CRISPR-based diagnostics, this approach enhances serotype discrimination, improves specificity, and offers a simple, rapid, and field-deployable solution for pathogen detection. Overall, this dual-channel biosensor provides a promising tool for clinical diagnostics, food safety monitoring, and infectious disease surveillance.

Keywords: biosensor, CRISPR/Cas13, dual-channel; one-pot; *E. coli*

6.2 Introduction

Escherichia coli is a highly diverse bacterial species, comprising both harmless commensals and pathogenic strains capable of causing severe disease.^{1, 2} Among them, *E. coli* O157:H7 is a major foodborne pathogen associated with outbreaks of hemorrhagic colitis and hemolytic uremic syndrome.³⁻⁵ The classification of *E. coli* relies on serotyping, which is determined by the combination of O (somatic), H (flagellar), and K (capsular) antigens.^{6, 7} However, the extensive

genetic and antigenic diversity within *E. coli* makes it challenging to differentiate pathogenic serotypes from nonpathogenic ones.^{8, 9} Distinguishing *E. coli* O157:H7 from closely related serotypes remains a critical issue in diagnostics, necessitating more precise and efficient detection strategies to improve food safety and public health monitoring.

Several methods have been developed for the detection of *E. coli* O157:H7. Culture-based methods, such as the use of selective media like Sorbitol MacConkey agar,^{10, 11} allow for the identification of *E. coli* O157:H7 based on its inability to ferment sorbitol. However, the culture-based methods require overnight incubation (16–24 h), making them time-consuming and impractical for rapid diagnostics. Immunoassays, such as enzyme-linked immunosorbent assay (ELISA), can detect *E. coli* O157:H7 by targeting its O antigen.^{12, 13} While offering a faster alternative to culture-based methods, ELISA is prone to cross-reactivity, with studies reporting false positive results due to antigenic similarities with *E. coli* O15.¹⁴ Nucleic acid-based methods, such as polymerase chain reaction (PCR) and quantitative polymerase chain reaction (qPCR), provide excellent accuracy and sensitivity but rely on expensive equipment and complex procedures, limiting their use in field settings.^{15, 16} Therefore, a faster, more affordable, and easy-to-use detection method is urgently needed, one that maintains high sensitivity and specificity for on-site applications.

Recent advances in biotechnology have led to the development of CRISPR-based diagnostic systems, offering a powerful new approach for nucleic acid detection. CRISPR-based detection offers high sensitivity and specificity without requiring complex equipment, making it ideal for rapid diagnostics.^{17, 18} The CRISPR/Cas13 system, in particular, is highly programmable due to its customizable CRISPR RNA (crRNA), allowing precise recognition of target sequences.¹⁹ Once activated, Cas13 exhibits trans-cleavage activity, indiscriminately cutting nearby single-stranded RNA (ssRNA) probes to generate a detectable signal. A notable example is the SHERLOCK system, which combines recombinase polymerase amplification (RPA) with CRISPR/Cas13 for one-pot detection at 37°C, eliminating the need for bulky instrumentation while maintaining high accuracy.^{20, 21} However, despite these advantages, most CRISPR-based methods struggle with multiplex detection due to the indiscriminate trans-cleavage activity, making it challenging to distinguish multiple targets in a single reaction.

In this study, we developed a CRISPR/Cas13-mediated biosensor for multiplex detection of *E. coli* O157:H7. The biosensor integrates two distinct Cas13 channels: LwaCas13a-TEX and

PsmCas13b-FAM, enabling simultaneous detection of the *rfbE*_{O157} and *fliC*_{H7} genes without cross-reactivity. By leveraging their trans-cleavage preferences, LwaCas13a and PsmCas13b independently recognize their target genes, activating cleavage of single-stranded RNA (ssRNA) probes labeled with fluorophores (FAM or TEX) and quenchers. In the presence of *E. coli* O157:H7, LwaCas13a targets *rfbE*_{O157} RNA, generating a red fluorescence from the TEX-labeled probe, while PsmCas13b recognizes *fliC*_{H7} RNA, producing a green fluorescence from the FAM-labeled probe. To further simplify the procedure and enhance sensitivity, we integrated this dual-channel biosensor with recombinase polymerase amplification (RPA) into a one-pot biosensor. This biosensor was successfully applied to detect *E. coli* O157:H7 in fecal samples, demonstrating high specificity and sensitivity. Compared to other CRISPR-based biosensors, our system enables dual-gene detection with minimal complexity and no cross-reactivity, highlighting its potential for clinical diagnostics. We anticipate that this one-pot biosensor will provide a more flexible, universal, and accurate approach for pathogen detection, contributing to improved public health.

6.3 Materials and Methods

6.3.1 Materials

All primers and ssRNA probes were synthesized by Integrated DNA Technologies (Coralville, IA). The Q5 High-Fidelity 2X Master Mix, Ribonucleotide (rNTP) Solution Mix, T7 RNA polymerase, RNase Inhibitor, RNA Cleanup Kit, DNA Cleanup Kit, and RNA synthesis kit were purchased from New England Biolabs (Ipswich, MA). The Luria-Bertani (LB) medium was purchased from Research Products International (Mount Prospect, IL). The Recombinase Polymerase Amplification (RPA) kit was purchased from TwistDx (Cambridge, UK). All other chemicals were purchased from Fisher Scientific (Hampton, NH).

6.3.2 Bacteria sample preparation

E. coli O157:H7, *E. coli* O26 H11, *E. coli* O45 H2, *E. coli* O103 H11, and *E. coli* O121 H19 were provided by Dr. Kim Waterman from Virginia Tech. *E. coli* O157:H19, *E. coli* O157:H43, *E. coli* O157:H39, *E. coli* O157:H16, *E. coli* O8:H7, *E. coli* O55:H7, and *E. coli* O147:H7 were requested from Penn State *E. coli* Reference Center (University Park, PA). *E. coli* K12 (ATCC#25404) was purchased from The American Type Culture Collection (Manassas, VA). *E. coli* BL21 was purchased from Novogene (Sacramento, CA). All bacteria strains were cultured in

LB medium. The concentration of bacteria was determined through traditional plate-counting method.

6.3.3 Nucleic acid target and crRNA preparation

Both RNA target and crRNA were prepared through in vitro RNA transcription. The protocol followed the instruction from HiScribe T7 High Yield RNA Synthesis Kit. Briefly, the *rfbE*_{O157} gene and *fliC*_{H7} gene were amplified from *E. coli* O157:H7 using Q5 High-Fidelity 2X Master Mix. The purified DNA template was incubated with T7 RNA polymerase mix and NTP at 37°C overnight. The RNA was purified using Monarch RNA Cleanup Kit. The DNA templates of crRNA were ordered from IDT. The crRNA was synthesized similarly to the RNA target.

6.3.4 Protein expression and purification

The Cas13 nucleases expression and purification were carried out as previous description.²² In brief, the DNA sequences of LwaCas13a and PsmCas13b were cloned into pMBP backbone, respectively. The protein expression vectors were transformed into T7 express competent cells. After transformation, a single colony was inoculated into 10 mL of LB medium with 100 µg/mL ampicillin and culture at 37°C overnight. The next day, a 2.5 mL overnight culture was transferred into 500 mL of LB medium containing 100 µg/mL ampicillin. Once the optical density at 600 nm (OD₆₀₀) reached 0.5, the protein expression was induced by 0.5 mM IPTG, and the cells were cooled to 18°C overnight. Cells were harvested by centrifugation at 4,500 g for 30 minutes at 4°C. Cell pellets were stored at -80°C.

Protein purification was performed at 4°C. The cell pellet was resuspended in the NEBExpress *E. coli* Lysis Reagent and broken through sonification. Lysate was cleared by centrifugation at 10,000 g for 30 min. The supernatant was purified by Ni-NTA resin. To remove the MBP and His-tag, the purified product was incubated with TEV protease at 4°C overnight. The Cas nuclease was further purified by heparin column and then concentrated by ultrafiltration. The final product was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then stored at -20 °C until use.

6.3.5 CRISPR/Cas13-mediated biosensor

The LwaCas13-TEX channel and PsmCas13b-FAM channel were constructed to detect the RNA of *rfbE*_{O157} gene and *fliC*_{H7} gene, respectively. The LwaCas13a-TEX channel consisted of

LwaCas13a (25 nM), crRNA-1 (30 nM), ssRNA-TEX probe (250 nM), and 1x reaction buffer (20 mM HEPES, 60 mM NaCl, 6 mM MgCl₂, pH 6.8). The PsmCas13b-FAM channel consisted of PsmCas13b (25 nM), crRNA-2 (30 nM), ssRNA-FAM probe (500 nM), and 1x reaction buffer. The total reaction volume of each channel was 50 μ L. The reaction was performed on the plate reader (Biotek) with fluorescent measured every 1 min.

6.3.6 RPA-assisted one-pot biosensor

The RPA-assisted one-pot detection was performed on the 96-well plate. Each reaction contains a 78- μ L master mix and a 2- μ L bacterial lysate.²³ The 78- μ L reaction master mix included the following reagents: one TwistAmp pellet, 29.5 μ L rehydration buffer, 4 μ L rNTP mixture (25 mM each), 1 μ L T7 RNA polymerase (50 U/ μ L), 3 μ L LwaCas13a (0.5 μ M), 3 μ L PsmCas13b (0.5 μ M), 2 μ L crRNA-1 (1 μ M), 2 μ L crRNA-2 (1 μ M), 2.5 μ L ssRNA-TEX probe (5 μ M), 2.5 μ L ssRNA-FAM probe (10 μ M), 1 μ L RNase Inhibitor (40 U/ μ L), 1.2 μ L primer mixture (20 μ M), 2.5 μ L MgOAc (280 mM), 0.5 μ L MgCl₂ (500 mM), and 23.3 μ L H₂O. The bacteria were lysis at 95°C for 5 min and then added into the master mix. The reaction was incubated on the plate reader (Biotek) at 37°C for 1 h. The fluorescent was measured every 1 min.

6.3.7 Animal experiments

All mice experiments were performed in accordance with protocols approved by the University of California Riverside Institutional Animal Care and Use Committee. The BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME). *E. coli* O157:H7 was cultured in LB medium and harvested by centrifugation at 5,000 rpm for 5 min. The cell pellet was resuspended into PBS buffer. A total of 14 eight-week-old mice were randomized into experimental and control groups. Mice in the experimental group were gavaged with 200 μ L of *E. coli* O157:H7 (10⁸ CFU), while mice in the control group were treated with 200 μ L PBS. During the early stages of infection (0-12 h), the fecal samples were collected at 4, 8, 12, 24 h. After 24 h, fecal samples were collected every 24 h up to 168 h. All samples were stored at -20°C for further experiments.

6.3.8 Statistical analysis

All experiments were repeated at least three times. The data was expressed as mean $\pm$ standard deviation. Student t-tests were used to evaluate the statistical significance between the fluorescence intensity from control and experimental group. The GraphPad Prism was used for data analysis.

6.4 Results and Discussion

6.4.1 Design of CRISPR/Cas13-mediated biosensor

The principle of simultaneous dual-gene detection using the CRISPR/Cas13-mediated biosensor is illustrated in **Figure 6-1**. The detection process involves four key steps: target gene amplification, transcription of DNA to RNA, CRISPR/Cas13 recognition of target RNA, and fluorescent signal readout.

Based on genome analysis of *E. coli*, the *rfbE*_{O157} and *fliC*_{H7} genes were selected as detection targets. The *rfbE*_{O157} gene encodes a key component of the O-antigen, a distinguishing surface structure of *E. coli* O157:H7, while the *fliC*_{H7} gene encodes the H7 flagellar protein, essential for bacterial motility.^{24, 25} To amplify these targets, specific primer pairs were designed for conserved regions of *rfbE*_{O157} and *fliC*_{H7}, with forward primers incorporating a T7 promoter sequence for subsequent transcription. Following amplification, DNA amplicons were transcribed into RNA using T7 RNA polymerase, generating the substrates for the CRISPR-based reaction.

Cas13 nucleases exhibit distinct dinucleotide cleavage preferences, which influence their trans-cleavage activity. For instance, LwaCas13a preferentially cleaves between adenine (A) and uracil (U), whereas PsmCas13b favors cleavage between two adenine (A-A).²⁰ Leveraging these

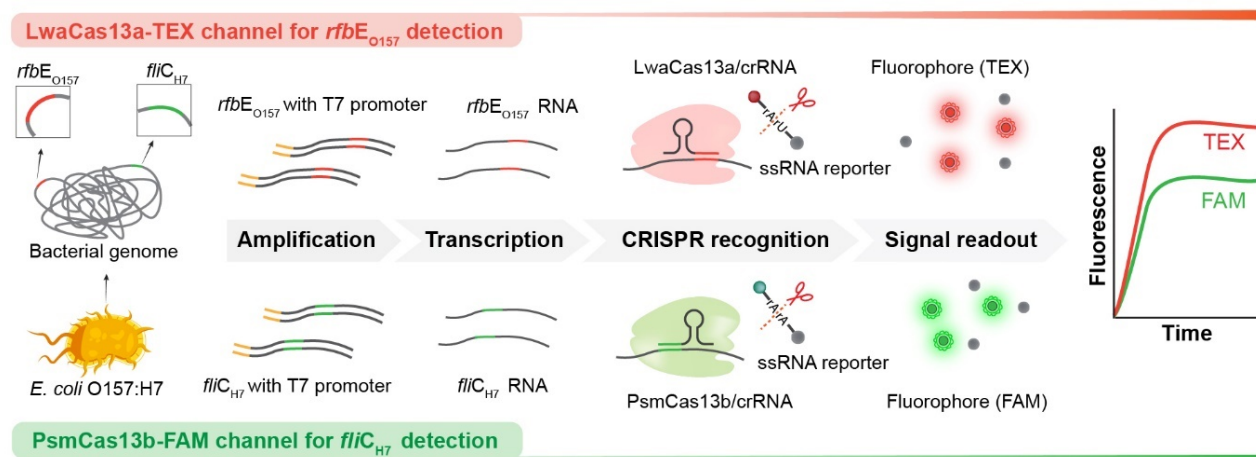


Figure 6-1 Schematic illustration of the CRISPR/Cas13-mediated biosensor.

The biosensor comprises two detection channels: the CRISPR/LwaCas13a channel for *rfbE*_{O157} and the CRISPR/PsmCas13b channel for *fliC*_{H7}. In the presence of *E. coli* O157:H7, the target genes are amplified from the bacterial genome and transcribed into RNA. Each Cas13 channel then recognizes its respective target RNA, activating Cas13-mediated trans-cleavage of the corresponding fluorescence probe (ssRNA-TEX for LwaCas13a, ssRNA-FAM for PsmCas13b), generating distinct fluorescence signals.

properties, two CRISPR/Cas13 channels were designed to specifically target *rfbE*_{O157} and *fliC*_{H7}. Each channel consists of a unique Cas13 protein, crRNA, and ssRNA reporter probe.

In the LwaCas13a-TEX channel, the crRNA was programmed to recognize *rfbE*_{O157} RNA, while the ssRNA reporter (TEX-TArArUGC-IAbRQSp) contained an A-U cleavage motif, flanked by a TEX fluorophore and a quencher. Upon target recognition, the LwaCas13a/crRNA complex cleaved the target RNA, triggering trans-cleavage of the ssRNA probe. This resulted in the release of the TEX fluorophore, generating a red fluorescence signal indicative of *rfbE*_{O157} detection. Likewise, in the PsmCas13b-FAM channel, the crRNA targeted *fliC*_{H7} RNA, triggering the cleavage of the A-A motif within the ssRNA-FAM probe (FAM-TArArAGC-IABkFQ) and generating a green fluorescence signal. By employing two distinct Cas13 nucleases with different sequence preferences, this dual-gene biosensor achieved high specificity while eliminating cross-reactivity between the two Cas13 channels (**Figure 6-1**).

6.4.2 Validation of two Cas13 channels

The CRISPR/Cas13-based biosensor consists of two independent channels: LwaCas13a-TEX for detecting *rfbE*_{O157} and PsmCas13b-FAM for detecting *fliC*_{H7}. Each channel comprises a Cas13 nuclease, a crRNA targeting the specific gene, and an ssRNA-fluorophore probe. LwaCas13a and PsmCas13b were purified using Ni-NTA chromatography and verified by SDS-PAGE, with observed molecular weights of 138.5 kDa and 155.4 kDa, respectively. The SDS-PAGE analysis confirmed expected protein sizes, with the PsmCas13b band appearing slightly higher than LwaCas13a. As shown in **Figures 6-2a and 6-2b**, the LwaCas13a-TEX channel corresponds to the red fluorescent of TEX, while the PsmCas13b-FAM channel corresponds to the green fluorescent of FAM. Fluorescence spectra analysis confirmed distinct emission peaks at 613 nm (TEX) and 520 nm (FAM) without spectral overlap, ensuring independent signal readouts for dual-gene detection.

To validate system functionality, a series of control tests were conducted in the absence of individual reaction components or target RNA. In reactions lacking target RNA, crRNA, or Cas13 nuclease, fluorescence intensity remained below 1500 a.u., indicating no digestion of the ssRNA probe. Similarly, no fluorescence was observed in the absence of the ssRNA probe. Only when all reaction components and the target RNA are present, a clear fluorescent signal is detected, with a 5-6-fold increase in fluorescence intensity over the background intensity.

Fluorescence kinetics were monitored for both channels (**Figure 6-2c, 6-2f**). Upon target RNA addition, fluorescence intensity was recorded every minute for 60 minutes. Both LwaCas13a-TEX and PsmCas13b-FAM channels exhibited rapid fluorescence increases, reaching a plateau within 15 minutes. Sensitivity analysis was performed by detecting varying target RNA concentrations (**Figures 6-2d, 6-2g**). In both channels, fluorescence intensity increased proportionally with target RNA concentration, yielding linear regression equations of $y = 6812x + 1925$ for LwaCas13a (TEX)

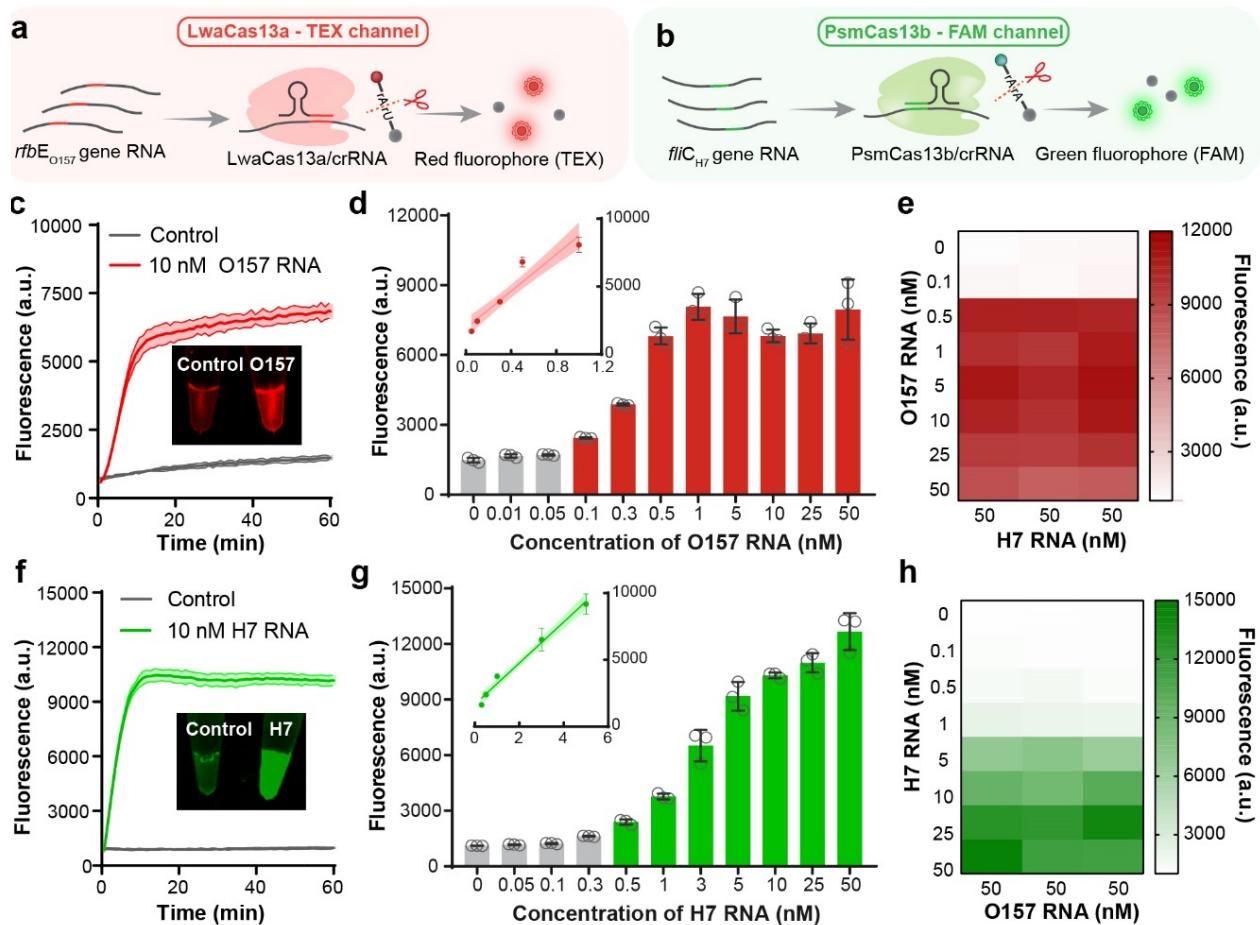


Figure 6-2 Validation of the CRISPR/Cas13 biosensor.

Schematic representation of the dual-channel CRISPR/Cas13 biosensor, where (a) the LwaCas13a-TEX channel targeted to the *rfbE_{O157}* RNA, and (b) the PsmCas13b-FAM channel recognized and cleaved *fliC_{H7}* gene. (c) Real-time fluorescence kinetics of the LwaCas13a-TEX channel upon addition of *rfbE_{O157}* RNA. (d) Sensitivity analysis of the LwaCas13a-TEX channel with varying target RNA concentrations. (e) Anti-interference test of LwaCas13a-TEX channel. (f) Real-time fluorescence kinetics of the PsmCas13b-FAM channel upon addition of *fliC_{H7}* RNA. (g) Sensitivity analysis of the PsmCas13b-FAM channel with varying target RNA concentrations. (h) Anti-interference test of PsmCas13b-FAM channel.

and $y = 1542x + 1676$ for PsmCas13b (FAM), where y represents fluorescence intensity (a.u.) and x represents target concentration (nM). The calculated LODs were 0.37 nM for *rfbE*_{O157} (LwaCas13a) and 1.08 nM for *fliC*_{H7} (PsmCas13b), demonstrating comparable sensitivity for both targets.

To evaluate anti-interference performance, each channel was tested with mixtures of *rfbE*_{O157} and *fliC*_{H7} RNA (**Figures 6-2e, 6-2h**). High concentrations of non-target RNA (50 nM) did not interfere with target detection, as fluorescence intensity remained consistent with individual target detection results (**Figure 6-2d, 6-2g**). This confirms that LwaCas13a and PsmCas13b function independently, eliminating cross-reactivity and enabling accurate dual-gene detection.

6.4.3 PCR-assisted dual-channel CRISPR/Cas13 biosensor

After evaluating the performance of each Cas13 detection channel, both channels were combined into a single reaction for the simultaneous detection of *E. coli* O157:H7 (**Figure 6-3a**). The control test confirmed that the two detection channels operated independently without cross-interference. To enhance sensitivity, PCR was used to amplify the target genes from bacterial samples. The PCR product was then added to a reaction tube containing transcription reagents and both Cas13 channels. With T7 RNA polymerase facilitating transcription, the resulting RNA was recognized by the CRISPR/Cas13 complexes, generating red and green fluorescence signals corresponding to the *rfbE*_{O157} and *fliC*_{H7} genes, respectively.

The sensitivity of the PCR-assisted CRISPR/Cas13-based biosensor was assessed using different concentrations of *E. coli* O157:H7 (**Figures 6-3b, 6-3c**). Both detection channels exhibited high sensitivity, achieving a limit of detection (LOD) of 43 CFU/mL. To evaluate specificity, the biosensor was tested against 14 bacterial strains (**Figure 6-3d**), including six *E. coli* non-O157:non-H7 strains (*E. coli* K12, *E. coli* BL21, *E. coli* O121:H19, *E. coli* O103:H11, *E. coli* O45:H2, *E. coli* O26:H11), four *E. coli* O157:non-H7 strains (*E. coli* O157:H19, *E. coli* O157:H43, *E. coli* O157:H39, *E. coli* O157:H16), three *E. coli* non-O157:H7 strains (*E. coli* O8:H7, *E. coli* O55:H7, *E. coli* O147:H7), and *E. coli* O157:H7. Fluorescence images were captured under different detection channels (**Figure 6-3e**), and fluorescence intensities were visualized as a heatmap (**Figure 6-3f**). The results demonstrated that red fluorescence was specifically generated in bacteria carrying the *rfbE*_{O157} gene, while green fluorescence was observed in those containing the *fliC*_{H7} gene. Merging TEX and FAM fluorescence images revealed a yellow signal exclusively for *E. coli* O157:H7, confirming successful dual-gene detection. Furthermore, the absence of

fluorescence in non-target strains validated the high specificity of the biosensor, with no cross-reactivity observed. To further validate these findings, PCR was performed to confirm the presence of the *rfbE*_{O157} and *fliC*_{H7} genes, and all CRISPR-based detection results were consistent with PCR results, demonstrating the accuracy and reliability of proposed method.

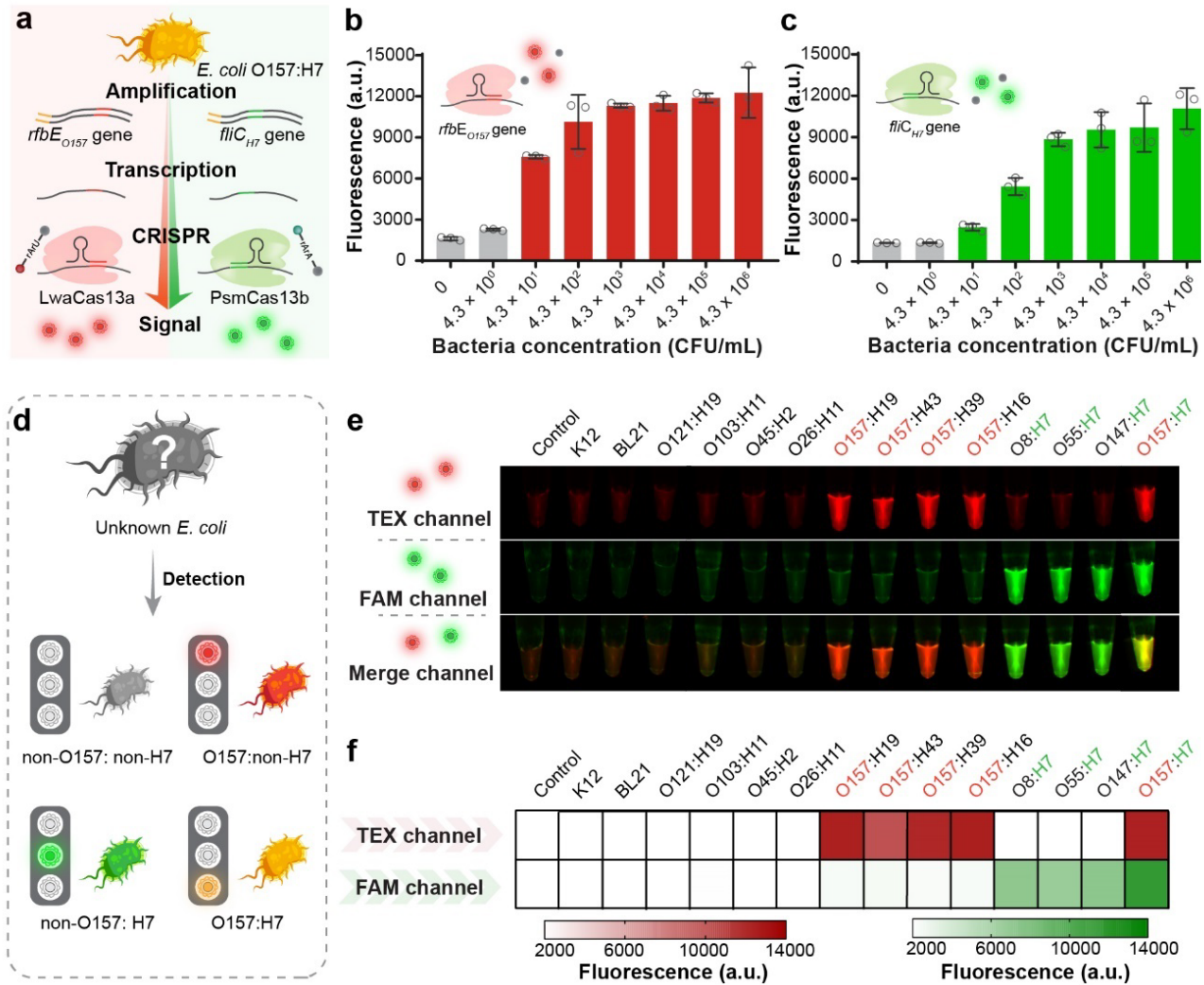


Figure 6-3 PCR-assisted dual-channel CRISPR/Cas13 biosensor.

(a) Schematic workflow of the PCR-assisted CRISPR/Cas13 detection system. The target genes (*rfbE*_{O157} and *fliC*_{H7}) were amplified by PCR, transcribed into RNA, and subsequently detected by the dual CRISPR/Cas13 channels. Sensitivity analysis of the (b) LwaCas13a channel and (c) PsmCas13b channel with varying concentrations of *E. coli* O157:H7. (d) Schematic drawing of specificity test, three channel from top to bottom: TEX channel, FAM channel, and merge channel. (e) Fluorescence images (f) and intensity of bacterial samples captured under TEX and FAM channels, showing specific red and green fluorescence signals corresponding to the presence of *rfbE*_{O157} and *fliC*_{H7} genes, respectively.

6.4.4 One-pot CRISPR/Cas13 biosensor

Although the dual-channel detection method integrating PCR with the CRISPR/Cas13 biosensor demonstrated high sensitivity and specificity, it remains unsuitable for on-site detection. First, PCR requires multiple temperature cycles, necessitating bulky equipment for precise thermal control. Second, the high amplification temperature is incompatible with downstream transcription and CRISPR reactions. To prevent thermal denaturation of Cas nucleases and T7 RNA polymerase, PCR-based detection must be performed in two separate tubes—one for amplification and another for CRISPR-based detection. This manual transfer of PCR products increases the risk of aerosol contamination, compromising assay reliability.

To overcome these limitations, recombinase polymerase amplification (RPA) was adopted as an alternative to PCR. RPA is an isothermal amplification method that operates efficiently at 37°C, making it fully compatible with subsequent transcription and CRISPR-based detection. The design of the RPA-assisted CRISPR/Cas13 biosensor is illustrated in **Figure 6-4a**. In this one-pot system,

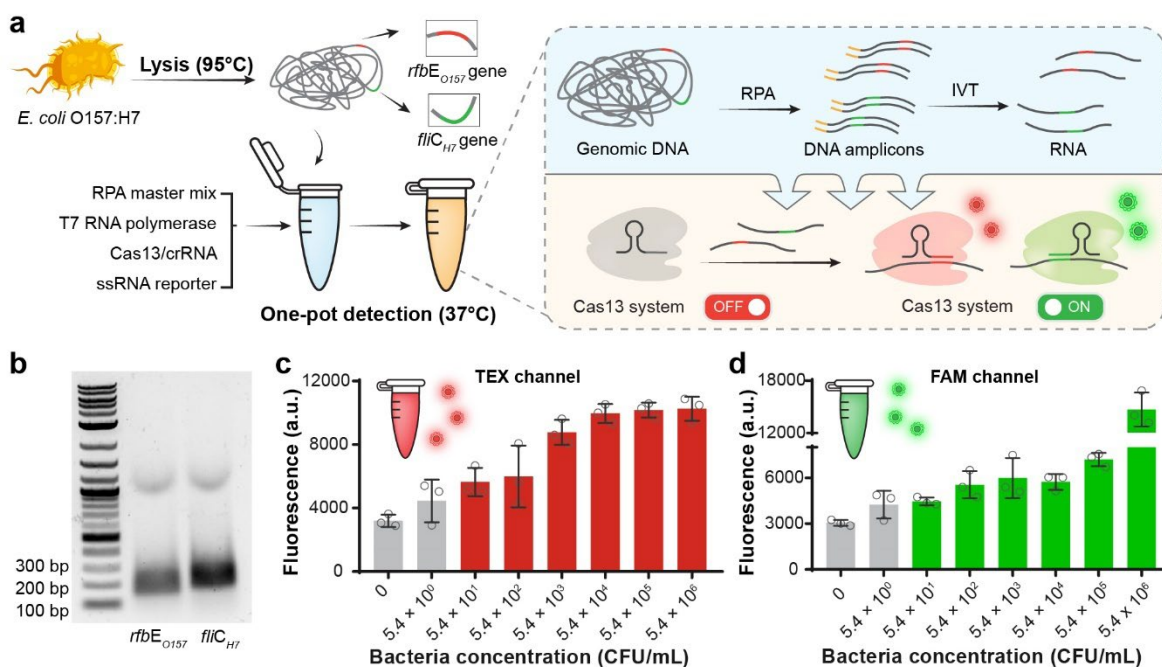


Figure 6-4 Development and validation of the one-pot CRISPR/Cas13 biosensor.

(a) Schematic illustration of the one-pot biosensor, integrating recombinase polymerase amplification (RPA), transcription, and CRISPR/Cas13-mediated detection within a single reaction tube at 37°C. (b) Gel electrophoresis analysis of RPA amplicons, confirming successful amplification of *rfbE*_{O157} (172 bp) and *fliC*_{H7} (191 bp) from bacterial lysates. (c, d) Sensitivity evaluation of the one-pot biosensor in TEX channel and FAM channel.

RPA reagents, T7 RNA polymerase, and CRISPR/Cas13 components were pre-mixed in a single reaction tube. Following cell lysis, the lysate was directly added to the prepared mixture, initiating isothermal amplification, transcription, and CRISPR-based detection at 37°C. This streamlined one-pot method eliminates the need for multiple reaction steps and reduces contamination risks, enhancing its potential for point-of-care applications.

The RPA amplicons of *rfbE*_{O157} and *fliC*_{H7} genes were characterized using gel electrophoresis. As shown in **Figure 6-4b**, the *rfbE*_{O157} amplicons were 172 bp, while the *fliC*_{H7} amplicons were 191 bp, confirming successful amplification from bacterial lysate. The sensitivity of the one-pot detection method was further evaluated (**Figure 6-4c, 6-4d**), revealing that both channels accurately detected their respective targets with a detection limit as low as 54 CFU/mL. Compared to the PCR-assisted CRISPR/Cas13 biosensor, the one-pot biosensor achieved similar sensitivity while offering greater user-friendliness and eliminating the need for bulky equipment. These advantages make the proposed approach more suitable for on-site detection, particularly in resource-limited settings.

6.4.5 Real sample application

E. coli O157:H7 is a major foodborne pathogen associated with severe gastrointestinal infections and life-threatening complications such as hemorrhagic colitis and hemolytic uremic syndrome (HUS). Transmission primarily occurs through contaminated food, water, or direct contact with infected animals, posing significant risks to public health. Accurate detection of *E. coli* O157:H7 in clinical samples is essential for effective disease surveillance and outbreak prevention. To evaluate the practical application of the proposed biosensor, a murine infection model was established, and fecal samples were collected for detection.

As illustrated in **Figure 6-5a**, experimental mice were orally administered a single dose of *E. coli* O157:H7 (10⁸ CFU) to establish the infection model. Following administration, fecal samples were collected at 0, 4, 8, 12, and 24 hours post-infection and subsequently every 24 hours until 168 hours (7 days). The presence of *E. coli* O157:H7 in each mouse was analyzed using the one-pot CRISPR/Cas13 biosensor, with fluorescent signals monitored simultaneously on the LwaCas13a-TEX and PsmCas13b-FAM channels (**Figures 6-5b, 6-5c**).

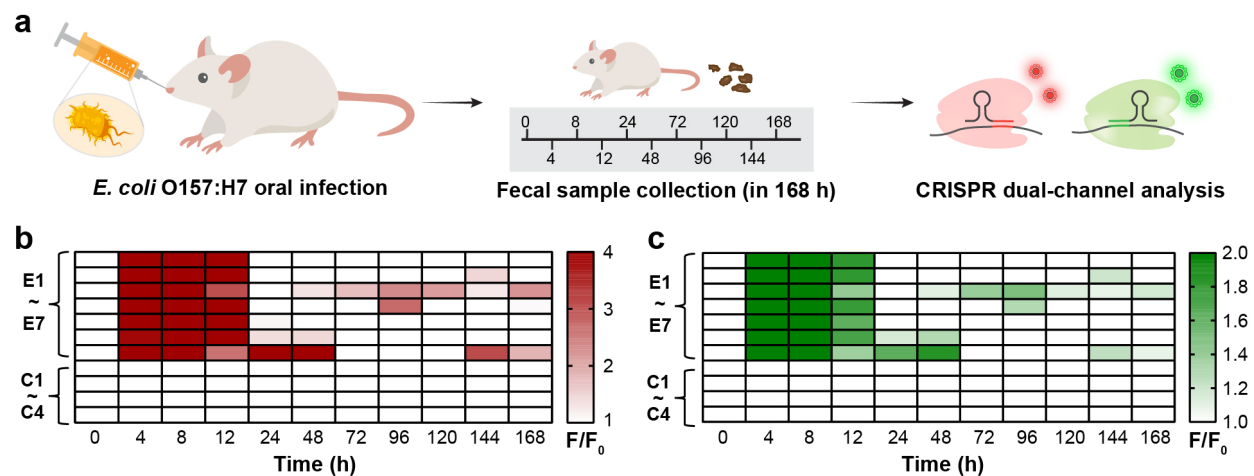


Figure 6-6 Detection of *E. coli* O157:H7 in fecal samples.

Detection of *E. coli* O157:H7 in fecal samples from a murine infection model using the one-pot CRISPR/Cas13 biosensor. (a) Schematic representation of the mouse infection model. Experimental mice were orally administered a single dose of *E. coli* O157:H7 (10^8 CFU), and fecal samples were collected at multiple time points (0, 4, 8, 12, and 24 hours post-infection, then every 24 hours up to 168 hours). (b, c) Fluorescence signal monitoring in the LwaCas13a-TEX and PsmCas13b-FAM channels, detecting *rfbE*_{O157} and *fliC*_{H7} genes, respectively.

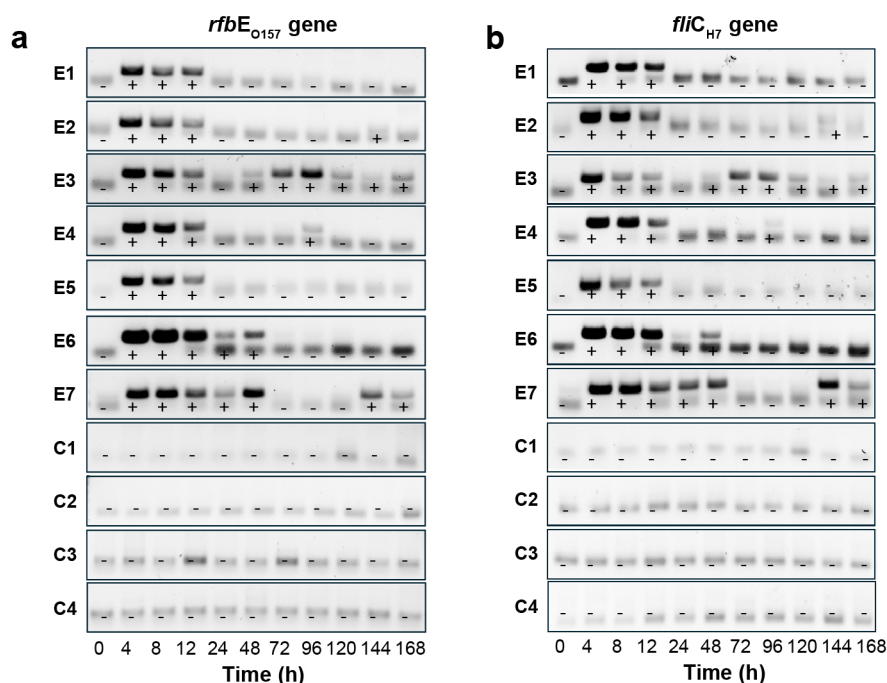


Figure 6-5 PCR results for mouse fecal samples.

Within the experimental group, infection dynamics varied among individual mice. Five out of seven mice (E1–E5) cleared *E. coli* O157:H7 within 24 hours, while two mice continued shedding the bacteria for up to 72 hours, suggesting active bacterial excretion. Interestingly, *E. coli* O157:H7 reappeared in four mice at later time points, first detected at 48 hours (Day 2), 96 hours (Day 4), or 144 hours (Day 6). These results indicate an incubation period of 2–6 days, consistent with previous studies on *E. coli* O157:H7 infection dynamics.

Furthermore, the detection results from the proposed biosensor were compared with PCR results (**Figure 6-6**), revealing identical infection patterns. This agreement validates the accuracy of the CRISPR/Cas13 biosensor and highlights its potential for clinical applications in pathogen detection.

6.5 Conclusion

Advances in CRISPR technology have introduced new approaches for clinical diagnostics. In this study, we developed a one-pot CRISPR/Cas13 biosensor for the simultaneous detection of two *E. coli* O157:H7 genes. Using the proposed biosensor, *rfbE*_{O157} and *fliC*_{H7} genes were amplified, transcribed, and cleaved within a single reaction tube, generating red and green fluorescence signals simultaneously. Compared to conventional CRISPR-based diagnostic methods, this biosensor leverages the unique trans-cleavage preferences of Cas13 nucleases to establish two independent fluorescence channels, enhancing serotype discrimination and reducing false positives.

To evaluate its real-world applicability, an *E. coli* infection model was established, and the biosensor was tested with fecal samples. The results demonstrated high reliability, showing strong agreement with standard PCR-based detection. Overall, the proposed dual-channel biosensor offers a highly sensitive, specific, and user-friendly platform for pathogen detection, paving the way for improved molecular diagnostics in clinical and field settings.

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Chapter 7

High-fidelity identification of single nucleotide polymorphism by type V CRISPR systems

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7.1 Abstract

Accurate and sensitive detection of single nucleotide polymorphism (SNP) holds significant clinical implications, especially in the field of cancer diagnosis. Leveraging its high accuracy and programmability, CRISPR system emerges as a promising platform for advancing the identification of SNPs. In this study, we compared two type V CRISPR/Cas systems (Cas12a and Cas14a) for the identification of cancer-related SNP. Their identification performances were evaluated by characterizing their mismatch tolerance to BRAF gene. We found that the CRISPR/Cas14a system exhibited superior accuracy and robustness over the CRISPR/Cas12a system for SNP detection. Furthermore, the blocker displacement amplification (BDA) was combined with the CRISPR/Cas14a system to eliminate the interference of the wild type (WT) and increase the detection accuracy. In this strategy, we were able to detect BRAF V600E as low as 103 copies with a sensitivity of 0.1% variant allele frequency. Moreover, the BDA-assisted CRISPR/Cas14a system has been applied to identify BRAF mutation from human colorectal carcinoma cells, achieving a high sensitivity of 0.5% variant allele frequency, which is comparable or even superior to most commercially available products. This work has broadened the scope of the CRISPR system and provided a promising method for precision medicine.

Keywords: CRISPR, BRAF V600E, Cas14, cancer diagnosis, single nucleotide polymorphism (SNP)

7.2 Introduction

Single nucleotide polymorphisms (SNPs) are genetic variations occurring when a single nucleotide changes in the genome. In the past few years, genome-wide association studies have

revealed that SNP mutations may directly affect human health by influencing DNA mismatch repair, cell cycle regulation, metabolism, and immunity.¹ Various SNPs have been reported to be associated with cancers.² A notable example is the V600E mutation in the BRAF gene, which is caused by the substitution of thymidine (T) with adenosine (A) and has been widely used as a biomarker for cancer diagnostics and treatment. This specific SNP mutation is tumorigenic to human cells by disrupting the normal growth cycle and interfering with extracellular signaling pathways. Therefore, the BRAF V600E mutation is often regarded as a biomarker of aggressive tumor growth in different cancers, such as metastatic melanoma, papillary thyroid cancer, and colorectal cancer.^{3, 4}

Due to its exceptional programmability and high sensitivity, the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) system has attracted much attention in the recent years.⁵ The type V CRISPR system (including Cas12a, Cas12b, Cas12f, etc.), renowned for its precise DNA recognition and *trans* cleavage capability, stands out from the crowd of CRISPR systems.⁶⁻⁸ Currently, there have been only a few studies exploring the gene editing using the type V CRISPR nucleases in BRAF V600E mutation. For example, Cas12a has been exploited to achieve gene disruption of BRAF V600E in HEK294T cell.⁹ Later, the structure-guided protein engineering was utilized to improve gene editing efficiency of Cas12a for the BRAF V600E.¹⁰ Despite the type V CRISPR system has made some progress in BRAF-related studies, most of them are mainly focused on *in vivo* applications, while the *in vitro* identification of BRAF mutation has not been explored.

Here, we explored the potential of two type V CRISPR systems, CRISPR/LbCas12a and CRISPR/UnlCas12f (CRISPR/Cas14a), for the identification of BRAF V600E mutation. The two CRISPR systems share similar sensing elements: CRISPR nuclease, guide RNA (gRNA), and ssDNA fluorophore-quencher (FQ) probe. Both systems follow an analogous sensing mechanism. In the presence of BRAF V600E, the nuclease/gRNA complex binds to the target, triggering the nuclease's *cis*-cleavage ability of DNA targets. Subsequently, the CRISPR systems indiscriminately cleave (*trans*-cleavage) the ssDNA FQ probes, releasing the fluorophores from the quenchers and generating a fluorescence readout. Therefore, the fluorescence intensity can accurately visualize the response of CRISPR system to the DNA target. Through investigating the performance of two type V CRISPR systems, we aim to establish the foundational knowledge for

the mechanism of CRISPR system and expand the potential applications of type V CRISPR nucleases in precision medicine.

7.3 Materials and Methods

7.3.1 Materials

All DNA oligonucleotides and crRNAs were purchased from Integrated DNA Technologies (Coralville, IA). The PCR master mix, RNA Synthesis Kit and RNA cleanup kit were purchased from New England Biolabs (Ipswich, MA). The DNA extracted from human colorectal carcinoma cells was purchased from Horizon Discovery (Waterbeach, UK).

7.3.2 Protein expression and purification

The pMBP-LbCas12a plasmid (Addgene #113431) and pLBH531_MBP-Cas14a1 plasmid (Addgene #112500) were gifts from Jennifer Doudna. The Cas enzymes expression and purification were carried out following the procedure with minor modifications. *E. coli* BL21(DE3) were transformed with the above plasmids and culture in LB broth at 37°C. Once the OD₆₀₀ of cell

Table 7-1 The sequences of gRNAs

Name	Sequence (5' to 3')
gRNA-1	UAAUUUCUACUAAGUGUAGAUGUCUAGCUACAG a GAAAUCUC
gRNA-2	UAAUUUCUACUAAGUGUAGAU u CUGUAGCUAGACCAAAAUCA
gRNA-3	CUUCACUGAUAAAGUGGAGAACCGCUUCACCAAAAGCUGUCCCUU AGGGGAUUAGAACUUGAGUGAAGGUGGGCUGCUUGCAUCAGCCUA AUGUCGAGAAGUGCUUUCUUCGGAAGUAACCCUCGAAACAAAUU CAUUUUUCCUCUCCAAUUCUGCACAAGAAAGUUGCAGAACCCGAA UAGACGAAUGAAGGAAUGCAACCUAGCUACAG a GAAAUCUCGA
gRNA-4	CUUCACUGAUAAAGUGGAGAACCGCUUCACCAAAAGCUGUCCCUU AGGGGAUUAGAACUUGAGUGAAGGUGGGCUGCUUGCAUCAGCCUA AUGUCGAGAAGUGCUUUCUUCGGAAGUAACCCUCGAAACAAAUU CAUUUUUCCUCUCCAAUUCUGCACAAGAAAGUUGCAGAACCCGAA UAGACGAAUGAAGGAAUGCAACUCGAGAUUUC t CUGUAGCUAG

The location of single nucleotide polymorphism is marked in red letter. The regions complementary to the target sequence are shown in bold black.

culture was reached 0.5, the cells were induced by 0.5 mM IPTG and grown overnight at 18°C. The cells were collected by centrifugation and resuspended in lysis buffer (50 mM Tris-HCl pH7.5, 500 mM NaCl, 0.5 mM PMSF, 1 mM TCEP, 0.25 mg/mL lysozyme, 5% glycerol). After breaking by sonification, the supernatant of cell lysis was filtered through 0.22 µm membrane, and then purified by Ni-NTA resin. The MBP and His-tag were removed with the enzyme digestion of TEV protease at 4°C overnight. The protein was further purified by heparin columns (GE, Hi-Trap) and then concentrated by ultrafiltration. The final product was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then stored at -20 °C until use.

7.3.3 sgRNA preparation

The sgRNA for Cas14a was prepared through *in vitro* transcription reactions. The dsDNA template of sgRNA was ordered from Twist Bioscience (San Francisco, CA) and incubated with T7 RNA polymerase at 37°C overnight using RNA Synthesis kit. All sgRNAs were purified with RNA cleanup kit. The sequences of all gRNAs are supplied in **Table 7-1**.

7.3.4 The mutation tolerance test of Type V enzymes

The mutation tolerance ability of Cas12a and Cas14a was evaluated by the fluorescence signal of each CRISPR/Cas system on the V600E at different variant allele frequency. The variant allele

Table 7-2 Target DNA and FQ probe

Name	Sequence (5' to 3')
WT_Top	CCTCACAGTAAAAATAGGTGATTTTGGTCTAGCTACAGTGAAATCTC
(ssDNA)	GATGGAGTG
WT_Bottom	CACTCCATCGAGATTTCACTGTAGCTAGACCAAATCACCTATTTTTA
(ssDNA)	CTGTGAGG
V600E_Top	CCTCACAGTAAAAATAGGTGATTTTGGTCTAGCTACAG ^a GAAATCTCG
(ssDNA)	ATGGAGTG
V600E_Bottom	CACTCCATCGAGATTTCT ^a CTGTAGCTAGACCAAATCACCTATTTTAC
(ssDNA)	TGTGAGG
FQ probe	/56-FAM/TTTTTTTTTTTT/3IABkFQ/

The location of single nucleotide polymorphism is marked in red letter.

frequency was calculated as the proportion of V600E in the V600E/WT mixture. The Cas12a reaction consisted of Cas12a (25 nM), crRNA (30 nM), ssDNA-FQ probe (50 nM), and NEBuffer2.1 (1×). The Cas14a reaction consisted of Cas14a (200 nM), sgRNA (250 nM), ssDNA-FQ probe (50 nM), and NEBuffer2.1 (1×). The total reaction volume of each system was 100 µL. The fluorescence signal of the reaction was measured every 1 min with a plate reader (BioTek Instruments, Winooski, VT). The target DNA and ssDNA-FQ probe were listed in **Table 7-2**.

7.3.5 Blocker displacement amplification (BDA)

The blocker of BDA was an oligo complementary to the BRAF wild-type sequence but phosphorylated at 3'-end. The forward primer (0.5 µM), reverse primer (0.5 µM), blocker (2.5 µM), and PCR master mix were mixed with the BRAF DNA template for BDA. The sequence of primers and blocker was listed in Table S3. The thermal cycling program started with 30 s initial denaturation at 98°C, followed by 35 cycles of 10 s at 98°C for denaturation, 30 s at 59°C for annealing, and 20 s at 72°C for extension. The BDA products were hold at 4°C until CRISPR/Cas14a reaction. The primers and blocker were listed in **Table 7-3**.

Table 7-3 Primers and blocker for blocker displacement amplification

Name	Sequence (5' to 3')
Forward primer	GATTTTGGTCTAGCTACAGa
Reverse primer	T*C*C*A*CAAAATGGATCCAGACAAC
Blocker	GCTACAGTGAAATCTCGATGG/3Phos/

*means phosphorothioate modification

7.3.6 BRAF V600E identification in human cell DNA

The BDA-assisted CRISPR/Cas14a system was used for identification of BRAF V600E in DNA extracted from human colorectal carcinoma cells. The BDA was performed as previously described. After that, 2 µL BDA product was added into a 98 µL premixed reagent (200 nM Cas14a, 250 nM sgRNA, 50 nM ssDNA-FQ probe, 2.5 U T7 exonuclease and 1×NEBuffer2.1) and incubated for 60 min at 37°C. The fluorescence signal was measured every 1 min (λ_{ex} : 485 nm; λ_{em} : 528 nm).

To eliminate the interference of baseline, the fluorescence signal of BDA-assisted CRISPR/Cas14a system was normalized by dividing the fluorescence intensity at the end point (F) by the initial fluorescence intensity (F_0).

7.3.7 Statistical analysis

Student t-tests were used to evaluate the statistical significance between the fluorescence intensity from the BRAF V600E the BRAF WT. One asterisk (*) and two asterisks (**) indicated the BRAF V600E group of data had a difference ($0.01 < p\text{-value} < 0.05$) and significant difference ($p\text{-value} < 0.01$) from the BRAF WT group, respectively.

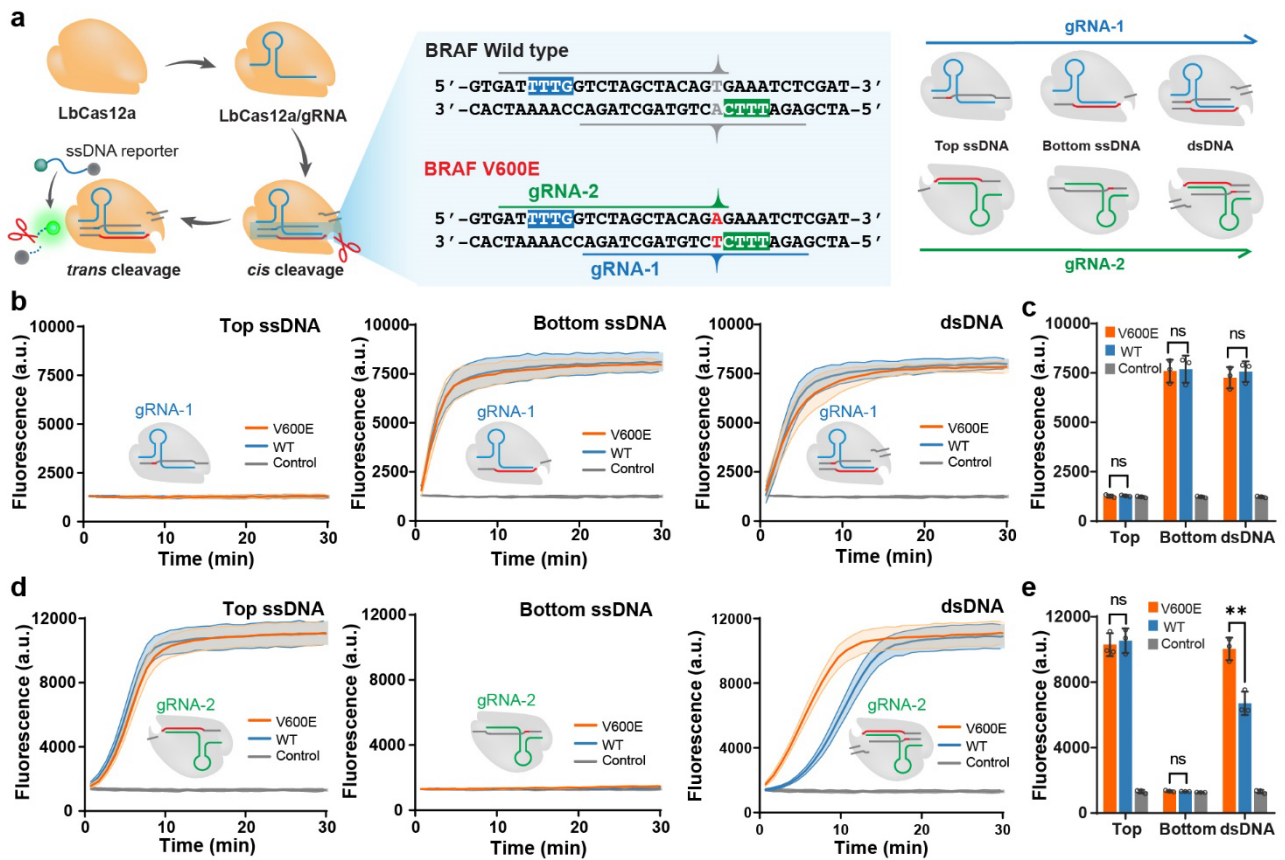


Figure 7-1 The detection principle of CRISPR/LbCas12a system.

(a) Schematic illustration of CRISPR/LbCas12a activated by three types of substrate DNA: top-strand DNA, bottom-strand DNA, and double-strand DNA. The gRNA-1 and gRNA-2 were designed to target bottom-strand DNA and top-strand DNA of BRAF V600E mutation, respectively. (b) Cleavage kinetics for LbCas12a/gRNA-1 system targeting top-strand DNA, bottom-strand DNA, and double-strand DNA. (c) The end-point fluorescence at 10 min of each type of DNA using LbCas12a/gRNA-1 system. (d) Cleavage kinetics for LbCas12a/gRNA-2 system targeting top-strand DNA, bottom-strand DNA, and double-strand DNA. (e) The end-point fluorescence at 10 min of each type of DNA using LbCas12a/gRNA-2 system. All ssDNA and dsDNA concentrations were 10 nM.

7.4 Results and Discussion

7.4.1 Feasibility of CRISPR/Cas12a system

As one of the most frequently used type V nucleases in CRISPR-based assays, we first chose LbCas12a to identify BRAF V600E. The LbCas12a system relies on a protospacer-adjacent motif (PAM) sequence to recognize the target region. As shown in **Figure 7-1a**, there are two PAM sites (5'-TTTN-3') near the BRAF mutation. Based on the PAM sites, two gRNAs were designed for the CRISPR system: gRNA-1 (target bottom-strand of V600E, mutation at 13th base of target region) and gRNA-2 (target top-strand of V600E, mutation at 1st base of target region). Furthermore, we tested the signal response of each LbCas12a/gRNA system toward V600E and wild type (WT) using ssDNA or dsDNA targets. The concentration of targets is 10 nM. The phosphate-buffered saline (PBS) without any DNA was used as a negative control. It was found that both systems generated a strong signal response on target ssDNA and dsDNA, and the fluorescence signal of V600E saturated within 10 min (**Figure 7-1b, d**).

Next, the end-point fluorescence at 10 min was utilized to investigate whether the LbCas12a systems have the ability to distinguish V600E from WT. The LbCas12a/gRNA-1 system has a strong cleavage activation toward bottom ssDNA and dsDNA targets, but there is no significant difference in fluorescence between V600E and WT (**Figure 7-1c**). On the contrary, the

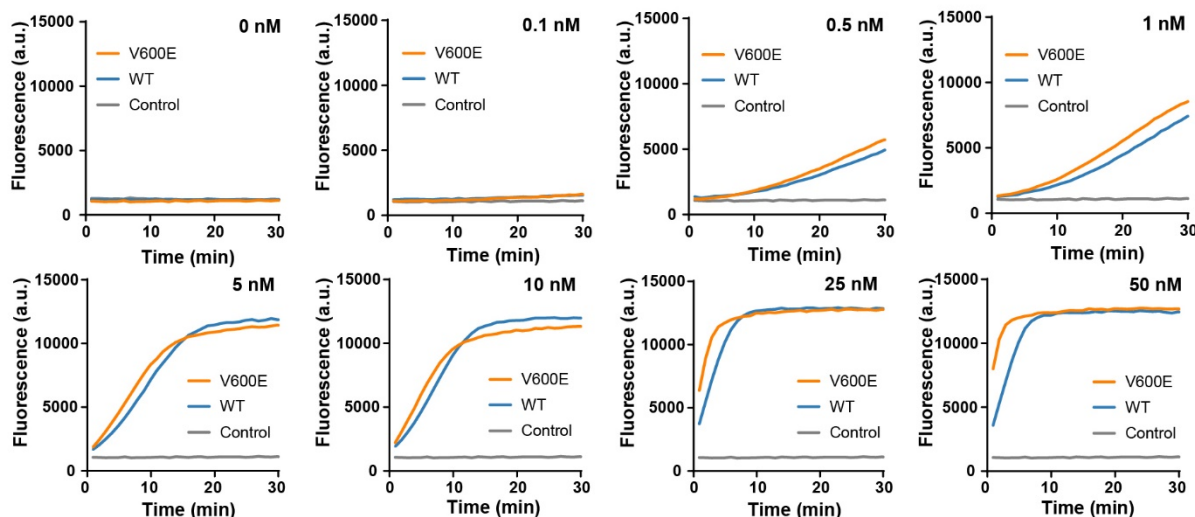


Figure 7-2 Optimization of LbCas12a/gRNA-2 mixture concentration.

The LbCas12a/gRNA-2 mixture concentration from low to high: 0, 0.1, 0.5, 1, 5, 10, 25, 50 nM. The target concentration is 0.5 nM.

LbCas12a/gRNA-2 generated a strong fluorescence response on top ssDNA and dsDNA targets (**Figure 7-1e**). It is found that there was a significant difference (p -value < 0.01) in dsDNA between V600E and WT during the first 10 min reaction. According to previous literatures, the CRISPR/LbCas12a system has lower mismatch tolerance within the initial 1st to 7th base of the target region from PAM, which can be used to discriminate the SNP between the mutation and WT.¹¹ Our results in **Figure 7-1e** confirm the previous conclusions, indicating that the LbCas12a system is more sensitive to the mismatch of dsDNA near the PAM site, and it is difficult for the LbCas12a system to distinguish the mismatch in ssDNA substrate. We further investigated that the LbCas12a/gRNA-2 system as a potential method to identify BRAF V600E mutation. To achieve a good detection performance, the concentration of LbCas12a/gRNA-2 mixture was optimized to 25 nM (**Figure 7-2**). And the limit of detection was determined to be 10 nM for the dsDNA target (**Figure 7-3a-c**). Afterward, we tested the LbCas12a/gRNA-2 system to detect V600E in a mixed solution of V600E and WT. V600E concentration was fixed at 0.5 nM and the WT concentrations were adjusted to achieve the different V600E/WT ratio ranging from 0.05% to 10%. It is interesting that all mixtures showed higher fluorescence signal than the pure V600E in the first 10 min (**Figure**

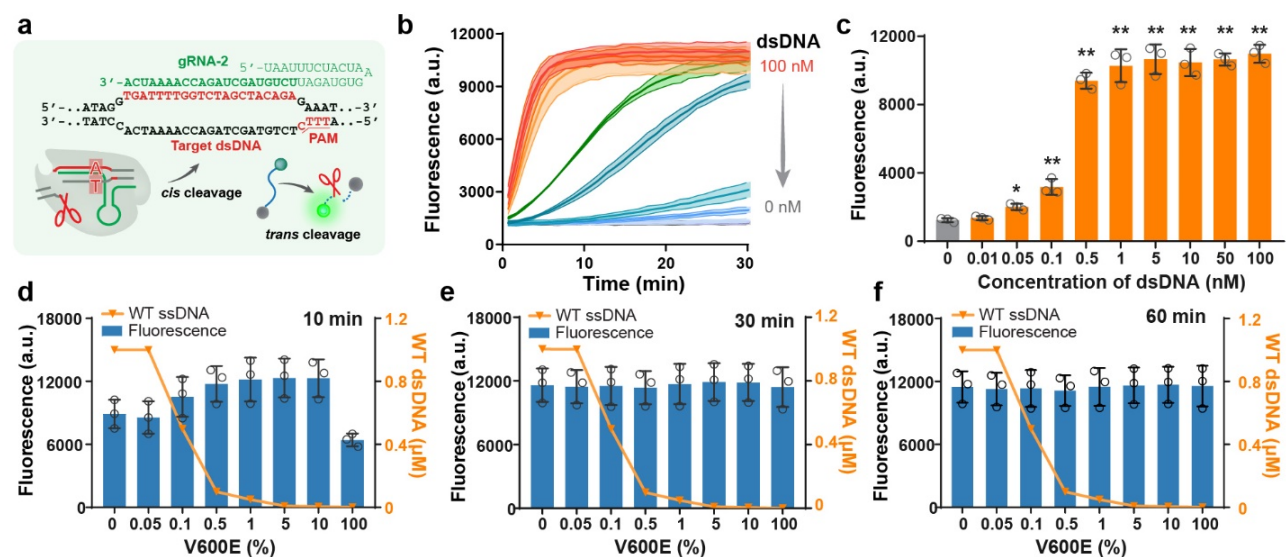


Figure 7-3 Detection of BRAF V600E dsDNA using LbCas12a/gRNA-2 system.

(a) Schematic illustration of Cas12a/gRNA-2 system. (b-c) The cleavage kinetics and end-point fluorescence at 10 min for Cas12a/gRNA-2 system toward different concentration of target dsDNA (The dsDNA concentration from low to high: 0, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 50, and 100 nM). (d-f) The end-point fluorescence of different ratio V600E/WT mixtures were detected by the LbCas12a/gRNA-2 system at 10, 30, and 60 min. The dsDNA of V600E was fixed at 0.5 nM.

7-3d-f), indicating the proposed LbCas12a/gRNA-2 system is not feasible to resist interference from WT. It is not an ideal system to be used for the V600E identification.

7.4.2 Feasibility of CRISPR/Cas14a system

To find a better CRISPR system for mutation identification, we turned our attention to a smaller type V nuclease – Cas14a. Different from the LbCas12a system, the Cas14a system possesses a PAM-independent property, which makes it more flexible in the design of gRNA. As shown in **Figure 7-4a**, two gRNAs were programmed for the CRISPR/Cas14a system: the gRNA-3 was aimed to match the bottom strand, while the gRNA-4 was designed to target the top strand.

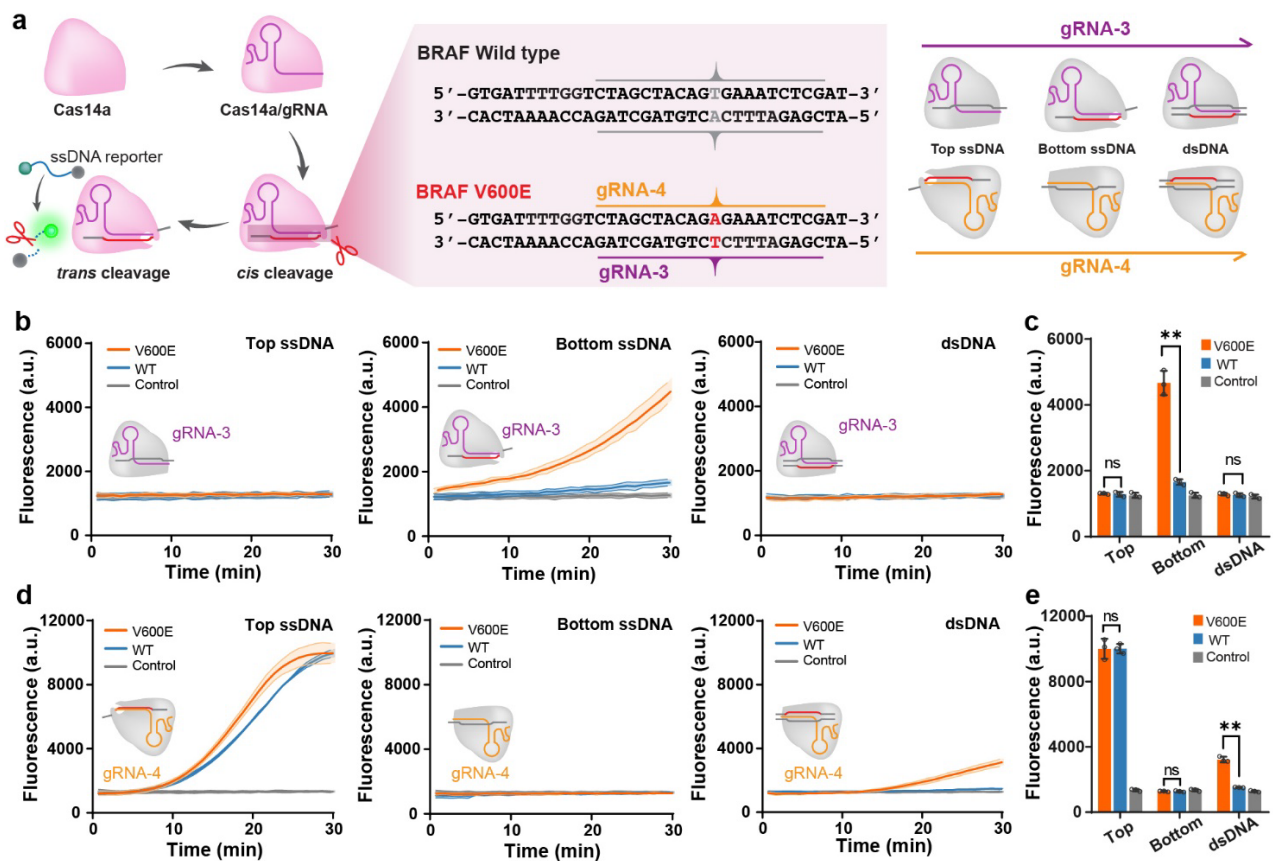


Figure 7-4 The detection principle of CRISPR/Cas14a system.

(a) Schematic illustration of CRISPR/Cas14a activated by three types of substrate DNA: top-strand DNA, bottom-strand DNA, and double-strand DNA. The gRNA-3 and gRNA-4 were designed to target bottom-strand DNA and top-strand DNA of BRAF V600E mutation, respectively. (b) Cleavage kinetics for Cas14a/gRNA-3 system targeting top-strand DNA, bottom-strand DNA, and double-strand DNA. (c) The end-point fluorescence at 30 min of each type of DNA using Cas14a/gRNA-3 system. (d) Cleavage kinetics for Cas14a/gRNA-4 system targeting top-strand DNA, bottom-strand DNA, and double-strand DNA. (e) The end-point fluorescence at 30 min of each type of DNA using Cas14a/gRNA-4 system. All ssDNA and dsDNA concentrations were 10 nM.

According to previous research, the Cas14a system has a lower mismatch tolerance when the mutation was in the middle of ssDNA target region.¹² To achieve higher sensitivity, both gRNAs were designed to contain the mutation site in the 11th base of the target region. Next, we investigated the cleavage kinetics of each Cas14a/gRNA system on ssDNA or dsDNA targets. Compared to Cas12a systems, Cas14a systems took a longer time to reach the plateau of fluorescence signal (**Figure 7-3b, d**). Within 30 min, the Cas14a/gRNA-3 system displayed a significant fluorescence difference between the V600E and WT on bottom ssDNA (**Figure 7-3c**), demonstrating its potential for further detection. On the contrary, the Cas14a/gRNA-4 system showed a higher mutation tolerance in the target sequence, resulting in a non-significant difference between V600E and WT after 30 min (**Figure 7-3e**). Cas14a/gRNA-4 system has a different but weak signal response on the V600E and WT dsDNA targets. This phenomenon can be explained by the Cas14a system can recognize dsDNA only in the presence of the PAM. But there is no PAM sequence designed in the downstream of our targets. Due to the weak fluorescence signals, the Cas14a/gRNA-4 system is not an ideal candidate for the V600E detection.

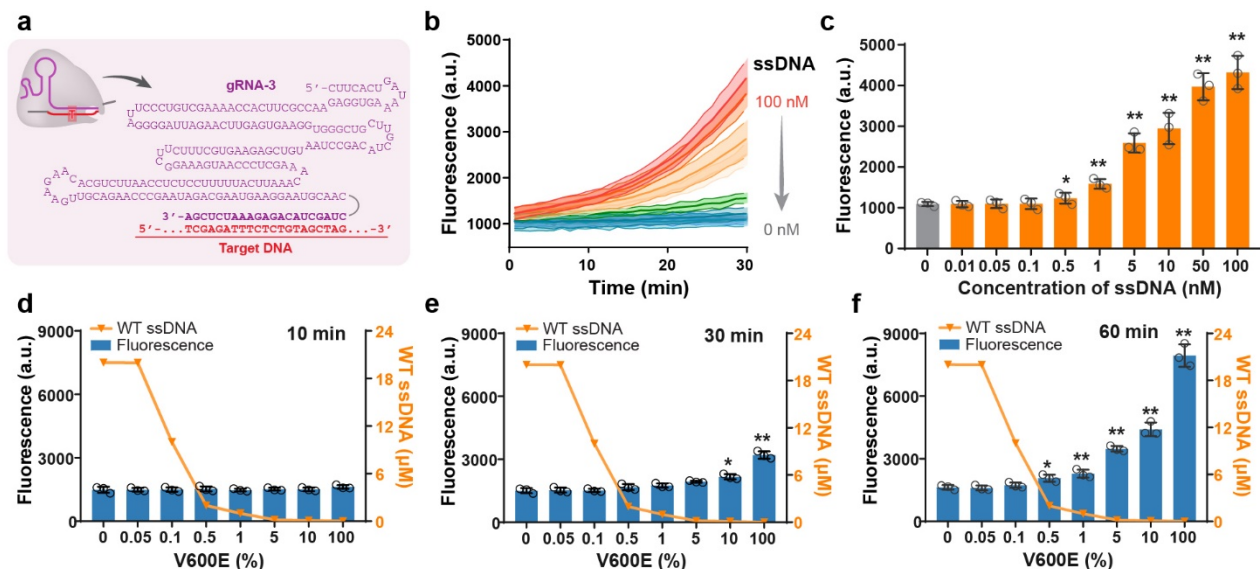


Figure 7-5 Detection of V600E bottom ssDNA targets using Cas14a/gRNA-3 system.

(a) Schematic illustration of Cas14a/gRNA-3 system. (b-c) The cleavage kinetics and end-point fluorescence at 30 min for Cas14a/gRNA-3 system toward different concentration of target ssDNA (The ssDNA concentration from low to high: 0, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 50, and 100 nM). (d-f) The end-point fluorescence of different ratio V600E/WT mixtures were detected by the Cas14a/gRNA-3 system at 10, 30, and 60 min, respectively. The concentration of V600E ssDNA was fixed at 10 nM.

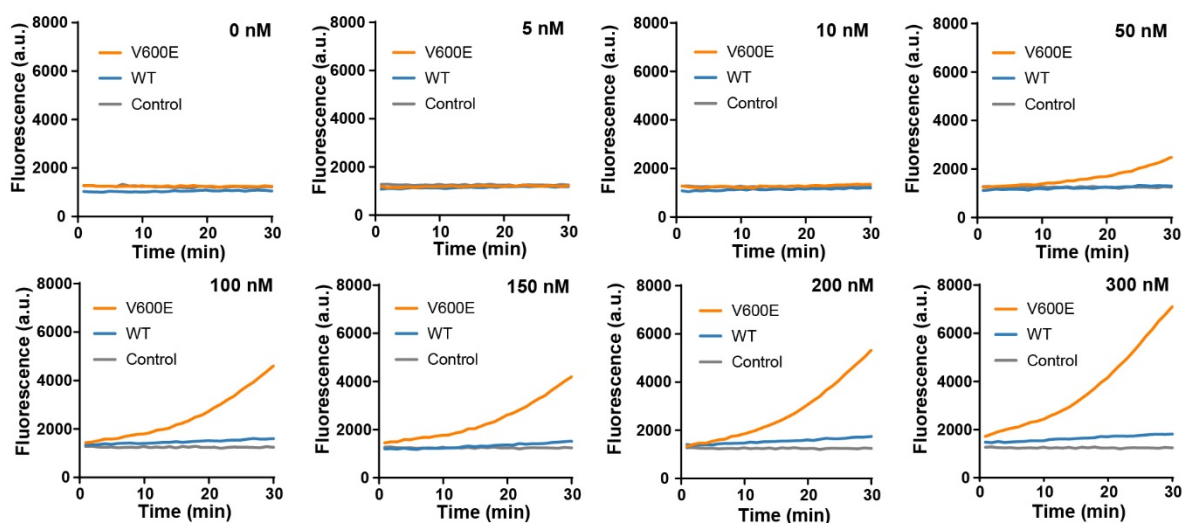


Figure 7-6 Optimization of Cas14a/gRNA-3 mixture concentration.

The Cas14a/gRNA-3 mixture concentration from low to high: 0, 5, 10, 50, 100, 150, 200, 300 nM. The target concentration is 10 nM.

7.4.3 Optimization of Cas14a/gRNA-3 system

Because of the ability to distinguish V600E from WT, the Cas14a/gRNA-3 system was further studied to detect bottom ssDNA targets (**Figure 7-5a**). The concentration of Cas14a/gRNA-3 complex was optimized to be 200 nM (**Figure 7-6**). The detection range of the Cas14a/gRNA-3 system was determined to be from 0.5 nM to 100 nM by measuring the fluorescence intensities corresponding to the bottom ssDNA targets at different concentrations (**Figure 7-5b-c**). Subsequently, the time required for the Cas14a/gRNA-3 system to reach a better sensitivity was also investigated. Various concentrations of WT were mixed with the ssDNA targets at 10 nM to obtain the variant allele frequency ranging from 0.05% to 10%. The Cas14a/gRNA-3 system was used to identify the V600E from the different mixtures. As shown in **Figure 7-5d-f**, the Cas14a/gRNA-3 can reach a detection limit of 0.5% variant allele frequency (fraction of V600E) in 60 min. Therefore, further CRISPR reactions were performed within 60 min and the corresponding end-point fluorescence intensities were collected.

7.4.4 Real sample application

In a clinic scenario, it is extremely challenging to detect the traces amount of SNP in tumor cells and blood. Moreover, a large number of WT may interfere with the accuracy of the detection assay due to the similarity between SNP and its WT. Therefore, we designed a blocker

displacement amplification (BDA) method combined Cas14a/gRNA-3 system to improve detection sensitivity and minimize WT interference. The BDA method is based on the hybridization affinity difference of forward primer between the V600E and WT templates, leading to an uneven amplification efficiency in each cycle.¹³ As shown in **Figure 7-7a**, the blocker oligo was completely complementary to the WT template and competed with the forward primer to bind the target template. On the other hand, because the blocker oligo has one base mismatch with the V600E template, the forward primer would easily displace the blocker and continue amplification in V600E template. According to this principle, the V600E and WT could be unequally amplified and then used for downstream CRISPR/Cas14a reactions. In our study, the reverse primer was modified by phosphorothioate (PT) at the 5' end, which can protect the bottom strand from degradation by T7 exonucleases and generate the bottom ssDNA as the target for the Cas14a/gRNA-3 system. The gel electrophoresis results indicated that the blocker effectively reduced the amplification of WT during BDA (**Figure 7-7b**). The BDA products were further used for CRISPR/Cas14a-based detection (**Figure 7-7c-d**). The fluorescence intensities of WT have a significant difference between the blocker-containing group and the blocker-free group, while the two V600E groups share similar signals. These results demonstrate the BDA visibly reduced the amount of WT in the products, which in turn minimizes the interference of WT on the downstream CRISPR assay. Besides, the amplification cycle was optimized to 40 cycles.

After constructing the BDA-assisted CRISPR/Cas14a, we tested the limit of detection and sensitivity of the proposed system for the V600E identification. To eliminate the influence of the baseline on the fluorescence signal, we adopted a normalization of the fluorescence signal. The synthetic V600E and WT dsDNA were amplified by BDA first, and then degraded by T7 exonucleases to generate bottom ssDNA. Leveraging the precise recognition capabilities of the Cas14a/gRNA-3 system, the V600E ssDNA can be identified as low as 10^3 copies (**Figure 7-7e**). When mixing 10^4 copies of V600E with different concentrations of WT, the sensitivity of detections can reach 0.1% variant allele frequency (**Figure 7-7f**), suggesting that the BDA-assisted CRISPR/Cas14 system can be utilized as an ideal method for BRAF V600E identification.

Moreover, to approach real-life clinical conditions, we tested the performance of the BDA-assisted CRISPR/Cas14a method to detect the BRAF gene extracted from the human colorectal carcinoma cells. Colorectal cancer is the third most common cancer and the leading cause of

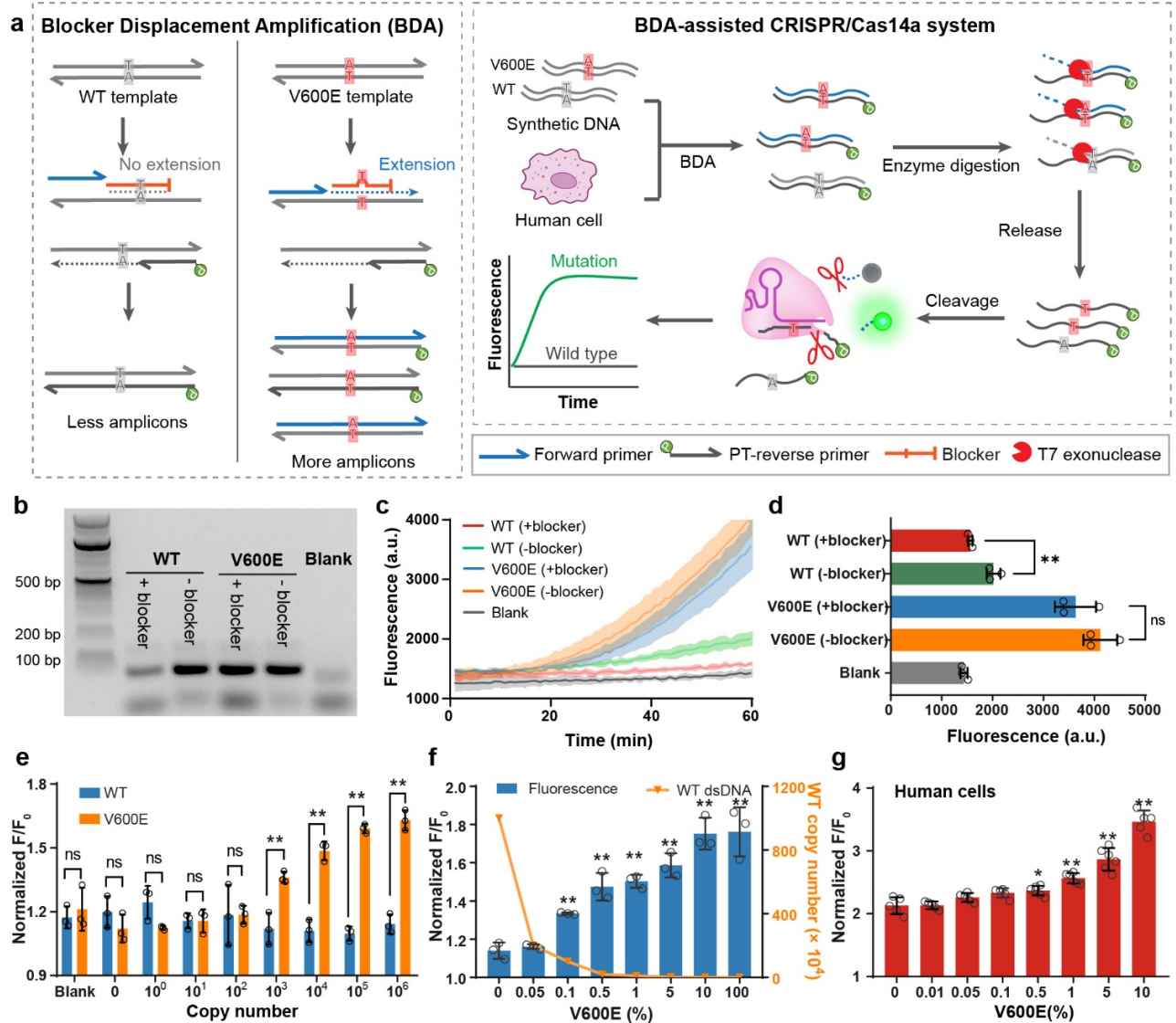


Figure 7-7. Real sample detection.

(a) Principle of blocker displacement amplification (BDA) and BDA-assisted CRISPR/Cas14a system. (b) Gel electrophoresis results of BDA for WT and V600E. (c-d) The cleavage kinetics and end-point fluorescence of BDA products. (e) The BDA-assisted CRISPR/Cas14a system for the detection of WT and V600E with different copy numbers. Normalized F/F_0 was calculated as the end-point fluorescence intensity divided by the initial fluorescence intensity. (f) Signal response of BDA-assisted CRISPR/Cas14a system towards different ratio of V600E/WT mixtures. The V600E was fixed to 10^4 copies. (g) The detection limits for the V600E in human cells using BDA-assisted CRISPR/Cas14a system.

cancer-related deaths worldwide. BRAF mutation occurs in around 10% of colorectal cancers.^{3, 14} Various concentrations of BRAF WT gene were mixed with BRAF V600E gene to obtain the variant allele frequency ranging from 0.01% to 10%. The mixtures were amplified using BDA to increase the fraction of BRAF V600E in the amplicons. Subsequently the BDA products were added into Cas14a/gRNA-3 system and generate fluorescence signal within 60 min. As shown in **Figure 7-7g**, the developed method can achieve a sensitivity of 0.5% for the V600E DNA in real samples. This sensitivity is much better than the most commercially available V600E identification kits, which normally have detection sensitivities above 1%.¹⁵ The insufficient sensitivity impeded the widespread adoption of early cancer screening and diagnosis. Therefore, our proposed method has great potential to be applied in the market for convenient and high-fidelity cancer screening and diagnosis in resource-poor areas.

7.5 Conclusion

In summary, we compared the analytical performance of two type V nucleases (Cas12a and Cas14a) for the identification of cancer-related SNP mutation (BRAF V600E). Although Cas12a offers higher reaction efficiency and lower detection limit, the PAM-dependent characteristic restricts the design of gRNA, and the high mutation tolerance makes it difficult to discriminate V600E from WT. On the contrary, taking the gift of PAM-independent characteristic and low mutation tolerance, the Cas14a nuclease is exceptionally well-suited for V600E identification. On this basis, we developed the BDA-assisted CRISPR/Cas14a system to achieve high-fidelity identification of BRAF V600E mutation. By leveraging unequal amplification, it becomes possible to diminish the WT interference on the subsequent CRISPR assay, thereby enhancing the sensitivity of BRAF V600E identification. The total detection time was less than 2 hours, which facilitates rapid cancer marker screening. The proposed detection system is comparable or even superior to some commercial SNP detection kits, which demonstrates the potential of CRISPR-based diagnosis technology in future biomedical diagnosis applications.

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Chapter 8

Conclusions and Prospectives

The emergence of CRISPR technology has opened up unprecedented possibilities for gene editing and molecular diagnostics. Building on its programmability and specificity, this dissertation presents a series of CRISPR-integrated biosensing platforms designed to enhance accuracy, sensitivity, and selectivity for different biomedical applications.

First, a CRISPR/Cas9-based phage engineering approach was established for high-throughput antimicrobial susceptibility testing (AST). This strategy enabled efficient phage genome editing and introduced a colorimetric biosensor for rapid detection of multidrug-resistant *E. coli*. Second, an electrochemical CRISPR/Cas12a biosensor was designed to detect *Salmonella* Typhimurium in poultry samples. The combination of PCR amplification and a hairpin-DNA-based interface led to high sensitivity and reliable detection in complex poultry matrices. Third, a SNP detection system was developed using Cas14a and blocker displacement amplification (BDA). This method accurately identified the cancer-relevant BRAF V600E mutation in both synthetic and real samples, outperforming several commercial kits. Fourth, a dual-gene, one-pot CRISPR/Cas13 biosensor was created for simultaneous detection of *E. coli* O157:H7 serotype markers. This system, validated in a mouse infection model, achieved multiplex detection in a single tube, making it suitable for clinical applications. Finally, a chemical strategy was introduced to enhance Cas12a-

Table 8-1 Comparison of different CRISPR/Cas systems in this thesis

System	Application	Strengths	Limitations
Cas9	Phage genome editing for AST in <i>E. coli</i>	Shortens detection time; enables high-throughput screening	Limited host range due to phage specificity
Cas12a	Electrochemical biosensor for <i>Salmonella</i> detection	Portable device; high sensitivity	Time-consuming electrode modification process
Cas12a-FSY	Enhanced sensitivity in SARS-CoV-2 detection	Strengthened Cas-crRNA binding; increased detection sensitivity	Complex protein expression and purification
Cas13	Dual-channel detection of <i>E. coli</i> O157:H7	High specificity; enables multiplexed detection	Potential cross-reactivity between detection channels
Cas14	High-fidelity SNP detection of BRAF V600E	High mismatch discrimination; PAM-independent targeting	Low enzymatic activity; extended reaction time

based biosensor sensitivity. Through site-specifically incorporating fluorosulfate-L-tyrosine into Cas12a, a covalent Cas12a-crRNA complex was formed. This modification improved trans-cleavage activity and detection of SARS-CoV-2, offering a new direction for CRISPR enzyme engineering. Comparisons among different CRISPR/Cas systems are shown in **Table 8-1**. Although significant progress has been made in the development of CRISPR-based biosensors, several key areas remain for further exploration and advancement (**Figure 8-1**).

Exploring potential properties of CRISPR Cas nucleases. The target recognition mechanism of the CRISPR-based detection mainly depends on the properties of the Cas nuclease. The emergence of artificial intelligence, particularly tools like AlphaFold, has facilitated the prediction and analysis of protein structures.¹ By integrating this technology with protein engineering techniques, it becomes possible to modify Cas nucleases and explore their potential properties. These modifications can improve the properties of Cas nuclease, such as high-temperature resistance, PAM-site versatility, and high recognition accuracy. Therefore, the combination of artificial intelligence tools and protein engineering may open new avenues for improving and customizing the functionalities of Cas nucleases, leading to sensitive, specific, and multiplexed detection of infectious diseases.

Establishing a standardized sample pretreatment process. While several CRISPR-based biosensors have already been developed for the detection of infectious diseases, a major challenge

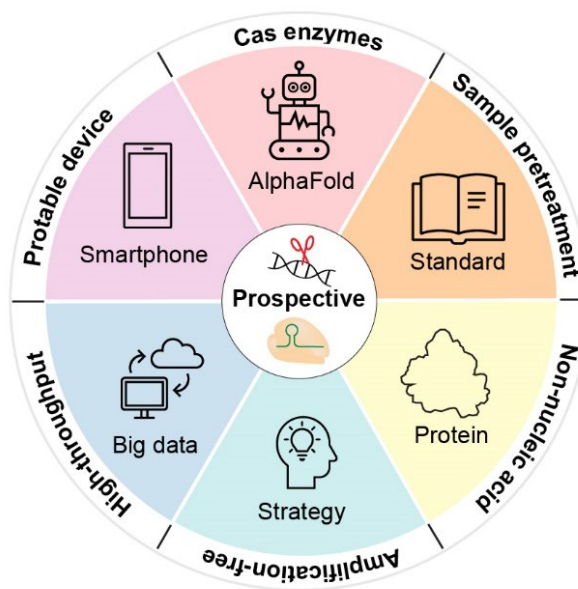


Figure 8-1 Future prospective in CRISPR-based biosensors.

is that there is no standardized procedure for sample pretreatment. Different pretreatment methods are required for infectious disease detection from environmental, food, and clinical samples. The lack of standardization makes it difficult to objectively compare the performance and reliability of different detection methods. Therefore, establishing a standardized sample pretreatment procedure is essential for accurate and consistent comparisons between different CRISPR-based biosensors and to facilitate the development of rapid detection.

Detecting non-nucleic acid targets. Currently, most CRISPR-based biosensor studies are centered around detecting nucleic acid targets. However, to expand the versatility of these biosensors, some researchers have focused on detecting non-nucleic acid targets, such as organic small molecules and proteins.²⁻⁴ A noteworthy challenge in developing activable CRISPR-based biosensors for non-nucleic acid targets is devising effective methods to translate the target recognition process into the collateral cleavage activity of Cas enzymes. In this context, aptamers^{2, 3} and bacterial allosteric transcription factors (aTFs)⁴ emerge as potential solutions for enabling the detection of non-nucleic acid targets using the CRISPR system. By leveraging the unique properties of aptamers and aTFs, researchers can design innovative approaches to facilitate non-nucleic acid target detection and expand the application of CRISPR-based biosensors.

Developing amplification-free strategies. The incorporation of nucleic acid amplification methods can significantly improve the sensitivity of CRISPR-based biosensors, but they also pose the risk of non-specific amplification and potential cross-contamination. Although several CRISPR-based biosensors have been developed for the amplification-free detection of infectious diseases, it is important to note that many of these methods are still in the laboratory validation stage.^{5, 6} This is mainly because the limited number of targets in the real sample makes it difficult to achieve sensitive and reliable detection using amplification-free CRISPR-based biosensors. Addressing this limitation is crucial for advancing the practical application of amplification-free CRISPR-based biosensors in real-world scenarios.

Improving detection at a high throughput manner. With the ongoing mutation of influenza viruses, the importance of high-throughput detection is becoming increasingly prominent. However, the vast amount of data generated from high-throughput assays present a challenge in terms of data analysis. In this context, machine learning offers a promising solution for effective analysis of such data. The use of machine learning algorithms can improve the accuracy of results, allowing discrimination between different mutant viruses.⁷ This application of machine learning

in the analysis of high-throughput assay data has the potential to improve our understanding of influenza virus mutations and contribute to more effective detection and surveillance strategies.

Integrating CRISPR-based biosensors onto portable devices. To overcome the limitations of sophisticated instrumentation for detection, the CRISPR-based methods can be integrated into portable devices, such as smartphones, portable electrochemical stations, and handheld Raman spectrometers.⁸ Most of these portable devices are equipped with their own batteries, which facilitate data collection and analysis directly in the field. Furthermore, the real-time uploading of data to the cloud through network connections enables infectious disease monitoring and facilitates the establishment of risk prediction models. Taking advantage of portable devices, the accessibility and applicability of CRISPR-based biosensors can be expanded, allowing them to be deployed in resource-limited settings and remote areas where sophisticated laboratory equipment is limited.

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