CRISPR-CAS SYSTEMS IN ANTI VIRAL AND ANTI MICROBIAL DEFENCE: RECENT ADVANCES, CHALLENGES AND BIODEFENSE IMPLICATIONS

By

Mubarrat Tazwar Bin Tarique
19136053

A thesis submitted to the Department of Mathematics and Natural Sciences (MNS) in partial fulfillment of the requirements for the degree of Biotechnology Program

Mathematics and Natural Sciences (MNS)
BRAC University
October 2025

Declaration

It is hereby declared that

1. The thesis submitted is my/our own original work while completing degree at BRAC University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. I have acknowledged all main sources of help.

Student's Full Name & Signature:

Mubarrat Tazwar Bin Tarique
19136053

Approval

The thesis/project titled "*CRISPR-Cas systems in anti-viral and anti-microbial defense; recent advances, challenges and biodefense implications*" submitted by Mubarrat Tazwar Bin Tarique (19136053) of Spring, 2019 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Science (BSc) in Biotechnology on 26th November, 2025.

Examining Committee:

Supervisor:
(Member)

Tushar Ahmed Shishir
Lecturer,
Department of Mathematics and Natural Sciences
BRAC University

Program Coordinator:
(Member)

Dr. Munima Haque
Director
Department of Mathematics and Natural Sciences
BRAC University

Departmental Head:
(Chair)

Dr. Firoze Hassanul Haque
Chairperson
Department of Mathematics and Natural Sciences
BRAC University

Abstract

CRISPR–Cas systems have evolved from a bacterial immune mechanism into a versatile platform for precision medicine and molecular diagnostics. This review synthesizes design principles and failure modes for three main application areas: (i) antivirals, where conservation-aware, multiplexed guides and delivery methods (AAV, LNP, or RNA RNP) are crucial for sustained suppression; (ii) precision antimicrobials, which allow for strain-specific killing or genetic disarming (chromosomal cuts, plasmid curing, CRISPRi) with minimal impact on commensal microbes; and (iii) Cas12/Cas13 diagnostics, promoting rapid, contamination-controlled, sample-to-answer workflows. In various use cases, we focus on specificity mapping (GUIDE-seq/CIRCLE-seq and related assays), escape management (including multiplexing, ortholog switching, and Acr awareness), and governance practices (such as assay hygiene, data provenance, and biosafety). We also address delivery challenges, matrix effects, and surveillance-driven retargeting as key factors influencing success. The aim is to create a playbook that guides choices of effectors, guide design and validation, delivery routes, and quality control measures, enabling the translation of programs from research to real-world deployment.

Keywords: CRISPR–Cas; antiviral therapeutics; precision antimicrobials; Cas12/Cas13 diagnostics; guide RNA design; drug delivery.

Acknowledgement

At the outset of this letter, I would like to express our deepest gratitude to Allah, the Creator, whose guidance and blessings have supported us throughout this academic journey.

I want to extend my most sincere appreciation to Dr. Firoze Hassanul Haque, Chairperson of the Department of Mathematics and Natural Sciences, and Dr. Munima Haque, Associate Professor, for their unwavering support, encouragement, and mentorship. Their guidance has been invaluable, and we are truly grateful for the knowledge and insights we have gained under their supervision.

Furthermore, I would like to express our sincere gratitude to my supervisor, Tushar Ahmed Shishir, who holds the position of Lecturer in the Department of Mathematics and Natural Sciences. Mr. Shishir's dedication, expertise, and continuous support played a pivotal role in shaping my thesis journey. His constructive feedback, patience, and willingness to share knowledge have been instrumental in my academic and professional development.

I am truly blessed to have had the opportunity to learn and grow under the guidance of such esteemed individuals. Their commitment to excellence and passion for education have left an indelible mark on our academic journey.

Table of Contents

List of Tables

List of Figures

List of Acronyms

AAV	adeno-associated virus
Acr	Anti-CRISPR protein;
ADA	Anti-Drug Antibody
ADR/AE	Adverse Drug Reaction / Adverse Event
AdV	Adenovirus (vector)
AIOD-CRISPR	All-In-One Dual CRISPR-Cas assay
ALT/AST	Alanine/Aspartate Aminotransferase
AMR	Antimicrobial resistance;
Amp-seq	Amplicon Sequencing
AsCas12a	Acidaminococcus sp. Cas12a
BE	Base Editing / Base Editor
BEP	Biosecurity Engagement Program
BLAST	Basic Local Alignment Search Tool
BLESS/BLISS	Breaks Labeling/In Situ Sequencing (DSB mapping)
blaCTX-M	ESBL β-lactamase family gene
blaNDM-1	New Delhi Metallo-β-lactamase-1
BSC	Biological Safety Cabinet
BSL-1/2/3/4	Biosafety Level 1–4
BTRP	Biological Threat Reduction Program
CARMEN	Combinatorial Arrayed Reactions for Multiplexed Evaluation of Nucleic acids
Cas	CRISPR-associated;
Cas12a (Cpf1)	CRISPR-associated protein 12a
Cas12b	CRISPR-associated protein 12b
Cas12f (Cas14)	Ultrafine Class 2 nuclease (aka Cas14
Cas13a/b/d	CRISPR-associated RNase family (Type VI)
CasΦ (Cas12j)	Miniature CRISPR nuclease from phag
CBE	Cytosine Base Edito
cccDNA	Covalently Closed Circular DNA
cGCP	Current Good Clinical Practicedf
cGMP	Current Good Manufacturing Practice
CFR	Code of Federal Regulations
CI	Confidence Interval
CIOMS	Council for International Organizations of Medical Sciences
CIRCLE-seq	Circularization for In vitro Reporting of Cleavage Effects by sequencing
CLSI	Clinical & Laboratory Standards Institute

Cq/Ct	Quantification/Cycle threshold (qPCR)
CRISPR	Clustered regularly interspaced short palindromic repeats;
CRISPRa	CRISPR activation;
CRISPRi	CRISPR interference;
CRISPR-CHIP	CRISPR-enabled graphene biosensor
CRISPR-FDS	CRISPR Fluorescence Detection System
CRISPResso	CRISPR edit outcome quantification toolkit
dCas	nuclease-dead Cas;
DETECTR	DNA Endonuclease-Targeted CRISPR Trans Reporter;
DETECTR BOOST	High-throughput Cas12 diagnostic workflow
Digenome-seq	Digested Genome Sequencing (off-target assay)
DISCOVER-seq	Detection of In Situ Cas Off-targets and Verification by sequencing
DNase	Deoxyribonuclease
dsDNA	Double-Stranded DNA
EBOV	Ebola Virus
EHS	Environment, Health & Safety
ELSI	Ethical, Legal & Social Implications
EUA	Emergency Use Authorization
EV	Extracellular Vesicle
Exo	Exosome
FDA	U.S. Food and Drug Administration
FELUDA	FnCas9 Editor Linked Uniform Detection Assay
FDR	False Discovery Rate
FISH	Fluorescence In Situ Hybridization
gDNA	Genomic DNA
GHSA	Global Health Security Agenda
GCP	Good Clinical Practice
GLP	Good Laboratory Practice
GUIDE-seq	Genome-wide, Unbiased Identification of DSBs Enabled by Sequencing;
GPU	Graphics Processing Unit
HCV	Hepatitis C Virus
HDR	Homology-directed repair;
HGT	horizontal gene transfer;
HHS	U.S. Department of Health and Human Services
HF-Cas9 / SpCas9-HF1	High-Fidelity Cas9 variants
HiFi Cas9	High-fidelity Cas9 (engineered variant)
HGT	Horizontal Gene Transfer
HIV-1	Human Immunodeficiency Virus-1
HLA	Human Leukocyte Antigen
HPV	Human Papillomavirus

HSV	Herpes Simplex Virus
HUDSON	Heating Unextracted Diagnostic Samples to Obliterate Nucleases;
IAC	Internal Amplification Control
IAV	Influenza A Virus
IDE	Investigational Device Exemption
IFN	Interferon
IND	Investigational New Drug
inhA	Isoniazid resistance target gene (M. tuberculosis)
IRB	Institutional Review Board
ISO	International Organization for Standardization
IVD	In Vitro Diagnostic
katG	Isoniazid resistance gene (M. tuberculosis)
LAMP	Loop-mediated isothermal amplification;
LDT	Laboratory-Developed Test
LFA	Lateral flow assay
LNP	Lipid nanoparticle
LoB	Limit of Blank
LoD	Limit of detection
LoQ	Limit of Quantitation
LV	Lentiviral Vector
MERS-CoV	Middle East Respiratory Syndrome Coronavirus
mecA	Methicillin resistance gene (MRSA)
ML	Machine Learning
MMEJ	Microhomology-Mediated End Joining
MRSA	Methicillin-Resistant Staphylococcus aureus
mRNA	Messenger RNA
NGS	Next-generation sequencing;
NHEJ	Non-homologous end joining;
NIST	National Institute of Standards and Technology
NPA	Negative Percent Agreement
NPV	Negative Predictive Value
NSR/SR	Non-Significant/Significant Risk (device studies)
PAM	Protospacer adjacent motif;
PBMC	Peripheral Blood Mononuclear Cel
PE	Prime Editing
PEI	Polyethylenimine
PEG	Polyethylene Glycol
pegRNA	Prime Editing guide RNA
PFS	Protospacer flanking site;
PI	Principal Investigator
PPE	Personal Protective Equipment
PPV	Positive Predictive Value
PoC	Point-of-care;
qPCR	quantitative polymerase chain reaction;

QC	Quality Control
QMS	Quality Management System
QOI	Quality of Information
RAA	Recombinase-aided amplification;
RNP	Ribonucleoprotein;
RNA-seq	RNA Sequencing
RPA	Recombinase polymerase amplification;
RSV	Respiratory Syncytial Virus
RT-LAMP	Reverse-transcription LAMP;
RT-PCR	Reverse-transcription PCR;
sgRNA	single-guide RNA;
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2
SHERLOCK	Specific High-sensitivity Enzymatic Reporter unlocking
SHINE	SHERLOCK and HUDSON Integration to Navigate Epidemics
SITE-seq	Selective Enrichment and Identification of Tagged genomic DNA Ends by sequencing
SOP	Standard Operating Procedure
SpCas9	Streptococcus pyogenes Cas9
SpG	PAM-relaxed SpCas9 variant (NGN)
SpRY	PAM-relaxed SpCas9 variant (NRN/NYN)
ssDNA	Single-Stranded DNA
ssRNA	Single-Stranded RNA
STOPCovid	SHERLOCK Testing in One Pot
T7 RNAP	T7 RNA Polymerase
tat	HIV-1 regulatory gene
TCR	T-Cell Receptor
TIDE/TIDER	Tracking of Indels by DEcomposition / with Reference
tracrRNA	Trans-activating CRISPR RNA
UDG	Uracil-DNA Glycosylase
UDI	Unique Device Identification
USAID EPT	USAID Emerging Pandemic Threats (program)
vanA / vanB	Vancomycin resistance operons
VLP	Virus-Like Particle
VRE	Vancomycin-Resistant Enterococcus

Chapter 1

Introduction

The discovery of clustered regularly interspaced short palindromic repeats (CRISPR) and their associated (Cas) proteins transformed modern biology, bridging microbial immunity and programmable gene manipulation (Jinek et al., 2012). Initially characterized as a bacterial defense mechanism against invading phages, the CRISPR–Cas system evolved into a versatile molecular toolkit capable of editing, silencing, or sensing nucleic acids across diverse biological contexts (Barrangou & Doudna, 2016; Koonin et al., 2017). Over the past decade, CRISPR-based technologies have advanced from laboratory proof-of-concepts to clinical applications and field-deployable diagnostics, redefining how pathogens are detected and controlled (Li et al., 2023).

1.1. Rationale and significance

Emerging and re-emerging infectious diseases continue to present significant challenges to global health systems. Antimicrobial resistance (AMR) and rapidly mutating viral genomes weaken the effectiveness of traditional treatments (World Health Organization [WHO], 2022). These evolving threats require adaptable countermeasures, precisely where CRISPR platforms excel, offering sequence-specific programmability, scalability, and modular design. Since a single guide RNA (gRNA) can re-target a Cas nuclease to new genetic sequences, the CRISPR system functions as a molecular "software" for biological "hardware," enabling quick responses to evolving pathogens (Hsu et al., 2014).

Unlike traditional antiviral or antibacterial drugs that act through biochemical inhibition, CRISPR technologies target nucleic acids directly, promising unparalleled specificity and reduced off-target toxicity (Chertow, 2018). Simultaneously, CRISPR-based diagnostics utilize the collateral cleavage activities of specific Cas effectors, such as Cas12 and Cas13, to convert nucleic acid recognition into measurable signals (Chen et al., 2018). Collectively, these applications situate CRISPR at the intersection of biotechnology, medicine, and global health security.

1.2. Historical emergence of the CRISPR era

The CRISPR journey began with prokaryotic observations in the late 1980s but crystallized as a gene-editing phenomenon after the 2012 demonstration of RNA-guided DNA cleavage (Jinek

et al., 2012). Subsequent discoveries expanded the CRISPR toolbox, introducing the Cas12 and Cas13 families, which possess single-strand-cutting activities and RNA-targeting capabilities, respectively (Abudayyeh et al., 2016; Zetsche et al., 2015). The identification of compact nucleases such as Cas14 and Cas12f further broadened prospects for viral targeting and therapeutic miniaturization (Harrington et al., 2018).

Within a decade, these developments enabled translational breakthroughs, most notably *in vivo* editing for transthyretin amyloidosis (Gillmore et al., 2021), and diagnostic Emergency Use Authorizations during the COVID-19 pandemic (U.S. Food and Drug Administration [FDA], 2020). Such milestones underscore CRISPR's rapid transition from microbial curiosity to cornerstone of molecular medicine.

1.3. Scope and objectives of this review

The primary aim of this review is to examine the expanding role of CRISPR–Cas systems in antiviral defense, antimicrobial development, and molecular diagnostics, while critically evaluating their biodefense implications and future potential. Unlike previous reviews that focus on either therapeutic or diagnostic aspects in isolation, this article integrates multiple dimensions, mechanistic principles, translational advances, biosafety challenges, and policy considerations, into a unified framework.

We emphasize three overarching questions:

1. How do distinct CRISPR–Cas effectors achieve specificity and efficiency across viral and bacterial targets?
2. What experimental and regulatory barriers constrain clinical and environmental deployment?
3. How can CRISPR-based systems be leveraged for real-time pathogen surveillance and global health security?

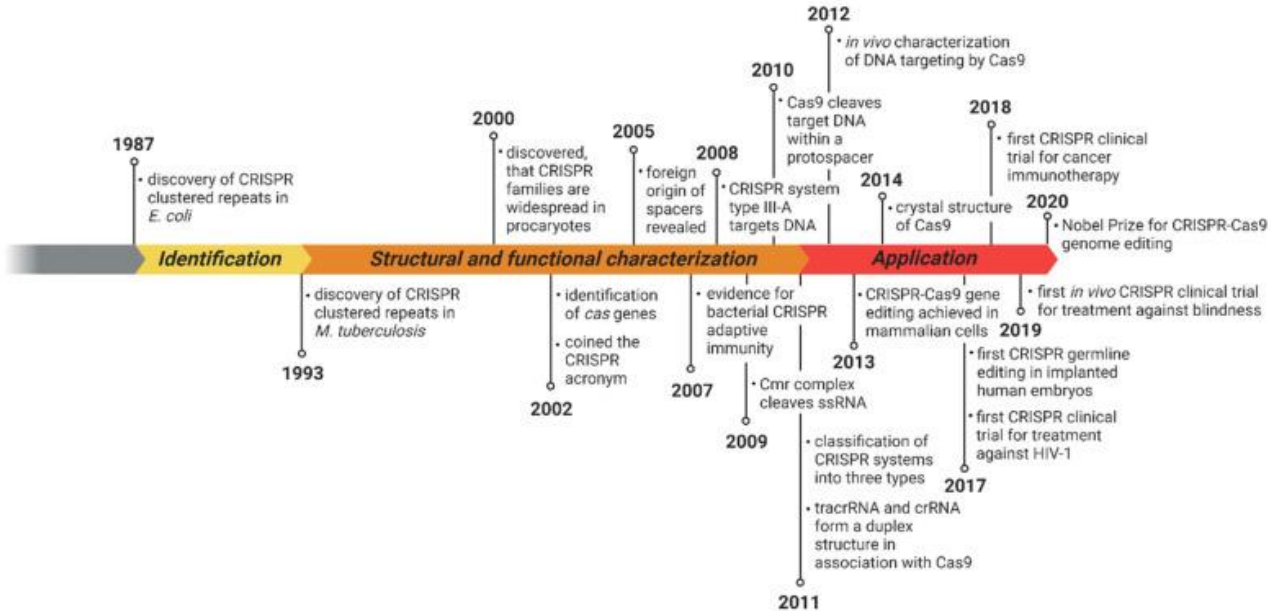

Figure 1 Timeline of key CRISPR–Cas milestones from initial discovery in 1987 to translational deployment in the 2010s. Adapted from Nidhi et al. (2021), licensed under CC BY 4.0

By synthesizing findings from molecular biology, synthetic biology, and global health literature, this review positions CRISPR as both a scientific tool and a biosecurity instrument, capable of transforming how humanity anticipates and mitigates biological threats (O'Neill, 2020; Koonin & Makarova, 2022).

1.4. Methodology and literature selection criteria

To ensure analytical depth and factual accuracy, we conducted a structured literature review following a modified systematic approach. Searches were performed through PubMed, Scopus, and Web of Science databases between January 2007 and September 2025. The query terms combined keywords such as CRISPR, Cas9, Cas12, Cas13, antiviral, antimicrobial, diagnostic, biosensing, and biodefense.

Studies were included if they:

1. Reported experimental or computational applications of CRISPR systems in viral or bacterial control;
2. Described CRISPR-mediated detection technologies or biosensing platforms;
3. Addressed biosafety, ethical, or policy implications of CRISPR deployment.

Excluded were non-peer-reviewed commentaries lacking primary data, redundant reviews, or speculative models without empirical validation. References were screened and cross-checked

to avoid duplication, ensuring representation of both seminal works (e.g., Jinek et al., 2012; Doudna & Charpentier, 2014) and recent advances up to 2025 (e.g., Li et al., 2023).

To maintain citation integrity, data were corroborated with official publications from regulatory authorities such as the World Health Organization (WHO) and the U.S. Food and Drug Administration (FDA), particularly regarding diagnostic authorizations and clinical trial registrations. Preprints were included only when they were subsequently peer-reviewed or when they provided unique experimental insights that were verified by later publications.

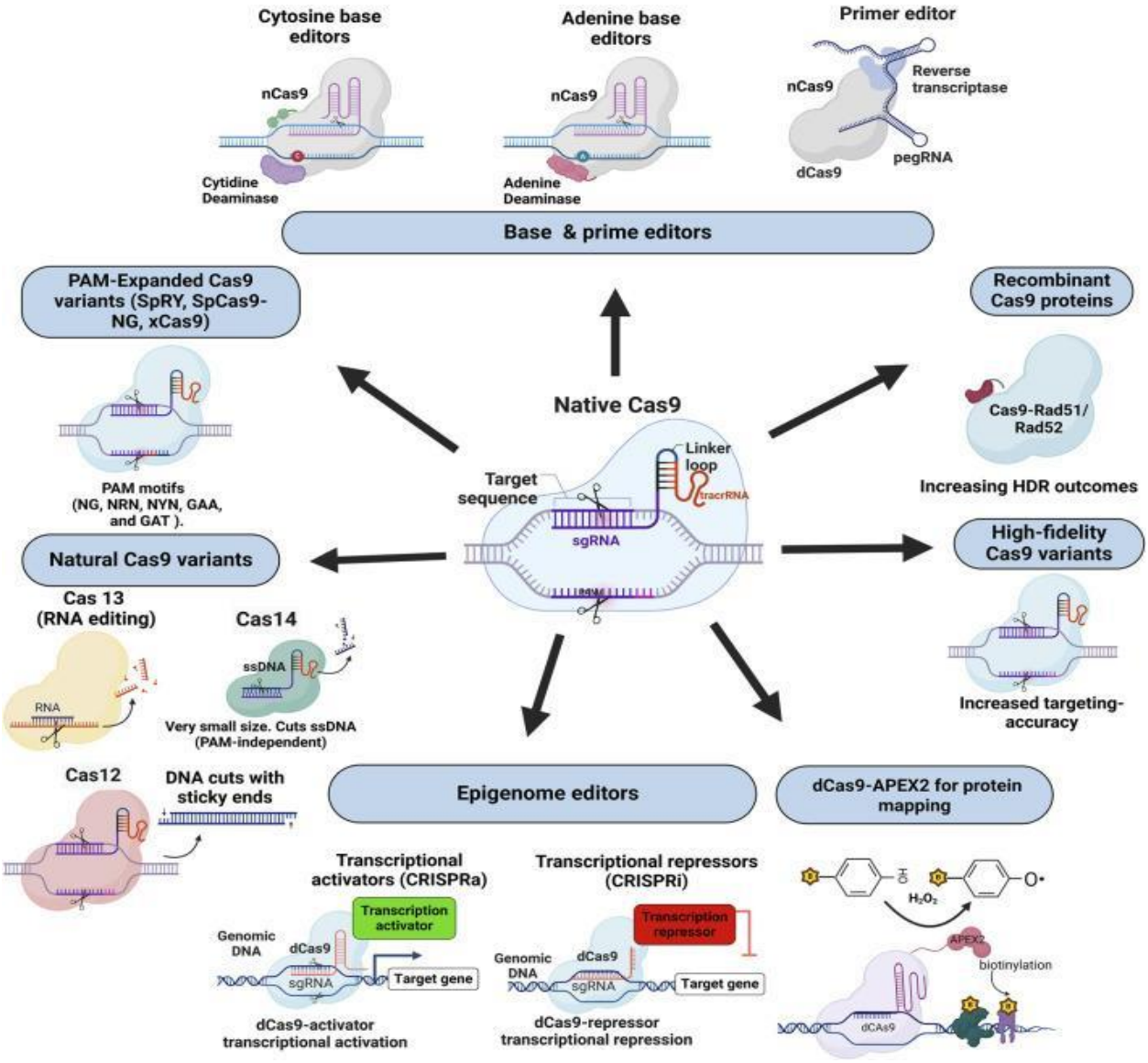

Figure 2 Conceptual overview of CRISPR–Cas functional modalities and engineered toolkits (Cas12, Cas13, Cas14; base/prime editors; CRISPRa/i/; high-fidelity and PAM-expanded variants) that map to genome and transcriptome manipulation for research and therapeutic applications. Adapted from Azeez et al. (2024), Frontiers in Genome Editing, CC BY 4.0

1.5. Structural overview

The review is organized into eight major sections.

- Section 2 introduces the molecular mechanisms and functional diversity of CRISPR–Cas systems.
- Sections 3 and 4 focus on antiviral and antimicrobial applications, emphasizing experimental progress and translational constraints.
- Section 5 explores CRISPR-based diagnostic platforms, including their scalability and field implementation.
- Section 6 discusses biological, ethical, and logistical challenges to clinical and environmental deployment.
- Section 7 examines the biodefense and governance aspects of CRISPR as a dual-use technology.
- Section 8 outlines emerging trajectories and interdisciplinary opportunities, integrating computational, AI-driven, and global surveillance perspectives.
- Finally, Section 9 synthesizes key takeaways and calls for a balanced roadmap integrating innovation with responsible stewardship.

As the world faces escalating threats from viral pandemics, AMR, and synthetic biology misuse, CRISPR–Cas systems emerge not merely as laboratory curiosities but as adaptive infrastructures for biological control. Their modularity allows near-real-time redesign of detection assays and therapeutic constructs in response to new pathogen sequences, an agility unmatched by conventional biotechnologies. Nevertheless, with this power arises the need for governance frameworks, delivery innovations, and equitable access to prevent uneven technological distribution and unintended misuse (Esvelt, 2023).

In this review, we aim to navigate these intersections, scientific, ethical, and strategic, so that CRISPR innovation contributes constructively to both medicine and global biosafety.

Chapter 2

CRISPR–Cas System: Molecular Foundations and Functional Diversity

The CRISPR–Cas system, originally discovered as a prokaryotic defense mechanism, exemplifies the coevolution between microbes and their mobile genetic invaders. Through a sophisticated process of sequence acquisition, transcription, and targeted interference, prokaryotes gain a form of adaptive immunity, enabling them to recognize and neutralize previously encountered genetic threats (Barrangou et al., 2007; Makarova et al., 2020). This microbial immune system's conceptual simplicity yet biochemical sophistication have made it one of the most transformative discoveries in molecular biology.

2.1. Classification and evolutionary landscape

Over evolutionary timescales, CRISPR–Cas systems diversified into two main classes and multiple types, distinguished by effector architecture and mode of target recognition (Shmakov et al., 2017).

Class 1 systems, Types I, III, and IV, utilize multi-subunit effector complexes composed of several Cas proteins. These complexes, such as Cascade (Type I) or Csm/Cmr (Type III), collaboratively recognize and degrade nucleic acids, a mechanism that offers robustness and multi-target flexibility. However, the need for multiple subunits complicates heterologous expression and limits their translational use (Makarova et al., 2020).

Class 2 systems, Types II, V, and VI, employ single, multidomain effector proteins that integrate all functional capacities within one molecule. This minimalist design, particularly exemplified by Cas9 (Type II), Cas12 (Type V), and Cas13 (Type VI), dramatically simplifies the manipulation and delivery of these proteins into eukaryotic cells (Jinek et al., 2012; Abudayyeh et al., 2016; Zetsche et al., 2015).

Beyond these canonical families, ongoing metagenomic mining continues to reveal novel and compact CRISPR effectors such as CasΦ, Casλ, and Cas12f (Cas14). These enzymes are microscopic (approximately 400–700 amino acids), making them compatible with viral delivery vectors like adeno-associated virus (AAV) and expanding therapeutic feasibility (Harrington et al., 2018; Pausch et al., 2020). Phylogenetic studies further suggest that many

of these effectors arose through horizontal gene transfer between bacteria and their viral predators, emphasizing CRISPR's dynamic evolutionary origins (Koonin et al., 2017).

This evolutionary adaptability, which mirrors an arms race between microbes and phages, underscores CRISPR's dual nature as both a natural immune memory and an engineerable molecular platform. Such duality enables it to bridge the gap between evolutionary biology and applied biotechnology seamlessly.

2.2. Mechanistic architecture and operational principles

The CRISPR immune cycle comprises three mechanistic stages, adaptation, expression, and interference, each mediated by distinct Cas protein functions.

Adaptation involves the integration of short DNA fragments, termed protospacers, derived from invading genetic material into the CRISPR array. The Cas1 catalyzes this step–Cas2 complex, which identifies foreign DNA and incorporates new spacers into the leader-proximal end of the array (Nunez et al., 2014). The result is a heritable record of past infections.

Expression begins when the CRISPR array is transcribed into a precursor CRISPR RNA (pre-crRNA), which is then processed into mature crRNAs, often aided by Cas6 or tracrRNA, depending on the system (Deltcheva et al., 2011). These crRNAs guide the effector complex to its matching target sequence.

Interference is the culmination of the immune process: Cas effectors, guided by the crRNA, recognize complementary nucleic acids through Watson–Crick base pairing. Recognition triggers nuclease activation, which cleaves the invader's genome and neutralizes the threat. PAM sequences adjacent to target sites act as "molecular fingerprints," ensuring discrimination between self and non-self DNA (Leenay et al., 2016).

This system achieves both memory and precision, providing a natural blueprint for programmable nucleic acid targeting in eukaryotic systems.

2.3. Structural and biochemical insights

Advances in structural biology have clarified how CRISPR systems achieve high fidelity. Cas9 adopts a bilobed structure, with a recognition (REC) lobe responsible for guide RNA binding and a nuclease (NUC) lobe containing the catalytic HNH and RuvC domains (Jiang & Doudna, 2017). Upon target engagement, conformational rearrangements align these domains, generating a double-strand break at the desired locus.

Cas12 effectors differ in that they cleave both DNA strands through a single RuvC domain, leaving characteristic staggered ends. Importantly, once activated, Cas12 and Cas13 exhibit collateral cleavage activity, indiscriminately cutting nearby single-stranded DNA or RNA molecules, respectively (Chen et al., 2018; Abudayyeh et al., 2016). This property, initially perceived as an anomaly, became foundational for CRISPR-based diagnostics, enabling nucleic acid detection through fluorescence or lateral flow readouts.

Furthermore, Cas13's RNA-targeting capacity offers unique therapeutic potential for modulating transcriptomes, combating RNA viruses, and transiently regulating gene expression without permanent genome alteration (Cox et al., 2017). Such mechanistic diversity demonstrates why CRISPR is not a single tool but a platform family adaptable across biological contexts.

2.4. Engineering and functional diversification

The engineering of CRISPR systems has extended their functionality far beyond genome cleavage. Nuclease-deactivated variants, such as dCas9 and dCas12, serve as programmable DNA-binding scaffolds that can recruit transcriptional activators, repressors, or epigenetic modifiers (Gilbert et al., 2014; Konermann et al., 2015). This has given rise to CRISPR interference (CRISPRi) and CRISPR activation (CRISPRa) systems capable of precise gene regulation at single-locus or genome-wide scales.

Further innovations include base editing, where Cas enzymes fused to deaminases introduce single-nucleotide changes without double-strand breaks (Komor et al., 2016), and prime editing, which combines reverse transcriptase activity with nickase Cas9 to install complex edits guided by a prime editing RNA (Anzalone et al., 2019). Collectively, these adaptations demonstrate that CRISPR is not limited to genome cutting but functions as a molecular programming framework capable of logic-like operations within living cells.

In parallel, delivery strategies have evolved from early plasmid transfections to advanced vectors, including lipid nanoparticles (LNPs), viral carriers, and extracellular vesicles. The successful in vivo application of LNP-encapsulated CRISPR components in clinical trials, notably the transthyretin amyloidosis study (Gillmore et al., 2021), marked a turning point for therapeutic genome editing.

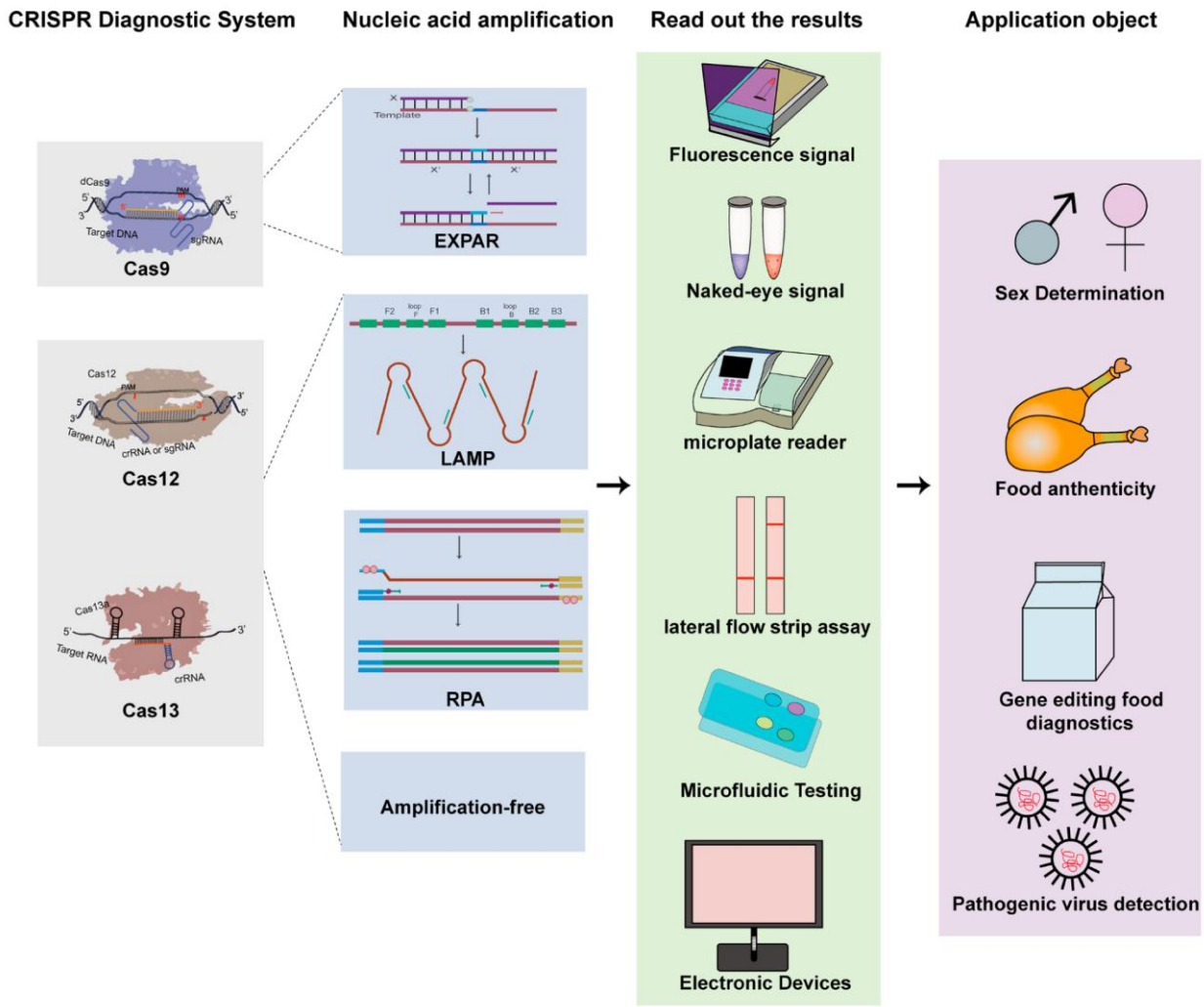

Figure 3 Comparative structural and functional properties of Cas9, Cas12, and Cas13 effector nucleases, including target type, PAM or PFS requirements, cleavage characteristics, and collateral activity. Adapted from Li et al. (2022), licensed under CC BY 4.0.

2.5. Fidelity, specificity, and safety considerations

While CRISPR's programmability underpins its power, it also poses risks of unintended cleavage at partially homologous sites. Such off-target events can lead to genomic instability or altered cellular phenotypes (Tsai & Joung, 2016). To mitigate these effects, high-fidelity Cas9 variants, eSpCas9, SpCas9-HF1, HypaCas9, and HiFi Cas9, have been developed, each incorporating rationally designed mutations that reduce non-specific DNA binding without compromising efficiency (Slaymaker et al., 2016; Chen et al., 2017).

Computational tools further enhance safety by predicting off-target potentials using thermodynamic models and machine learning algorithms trained on empirical data. These

improvements are essential for translational use, where regulatory agencies demand validated specificity profiles before clinical approval.

Equally critical is the management of immune responses to Cas proteins, particularly those derived from common human pathogens such as *Streptococcus pyogenes* and *Staphylococcus aureus*. Studies have detected pre-existing antibodies and T-cell responses against Cas9, necessitating the use of alternative effectors or transient delivery strategies to minimize immunogenicity (Charlesworth et al., 2019).

2.6. Conceptual significance and future outlook

The mechanistic and structural understanding of CRISPR–Cas systems provides the foundation for their antiviral, antimicrobial, and diagnostic applications. Each effector's biochemical traits, whether targeting DNA versus RNA, collateral activity, or PAM dependency, directly determine its suitability for various biomedical goals. Cas13's RNA focus aligns with antiviral interference, Cas12's collateral cleavage underpins diagnostics, and Cas9's precision editing drives therapeutic interventions.

In many ways, CRISPR mirrors the natural intelligence of microbial evolution: an adaptive learning process encoded in nucleic acid sequences. The continuous discovery of new Cas variants through metagenomics, coupled with advances in AI-driven guide design and delivery technologies, ensures that CRISPR will remain a living system of innovation. As its mechanistic repertoire expands, so too does its potential to redefine biological control and pathogen defense.

Table 1 Comparative properties of widely used CRISPR–Cas effectors.

Effector	Target Type	PAM / PFS Requirement	Cleavage Pattern	Collateral Activity	Delivery Compatibility	Primary Applications
Cas9 (Type II)	DNA	NGG (SpCas9) and related PAM variants	Double-strand break (HNH + RuvC domains)	No	AAV, LNP, plasmid, RNP	Genome editing, antiviral DNA targeting
Cas12a (Cpf1, Type V)	DNA	T-rich PAM (TTTV)	Staggered 5′ overhang cut	Yes (ssDNA)	LNP, RNP, minicircle vectors	Editing + DNA diagnostics (e.g., DETECTR)

Cas13 (Type VI)	RNA	PFS-dependent, no PAM	RNA cleavage only	Yes (ssRNA)	LNP, mRNA, RNP	Antiviral RNA knockdown + diagnostics (e.g., SHERLOCK)
CasΦ / Cas12j (ultra-compact)	DNA	Minimal / flexible PAM	Single-site dsDNA cleavage	Yes (system-dependent)	AAV, phage, nanoparticle	Miniaturized delivery, compact editors, field diagnostics

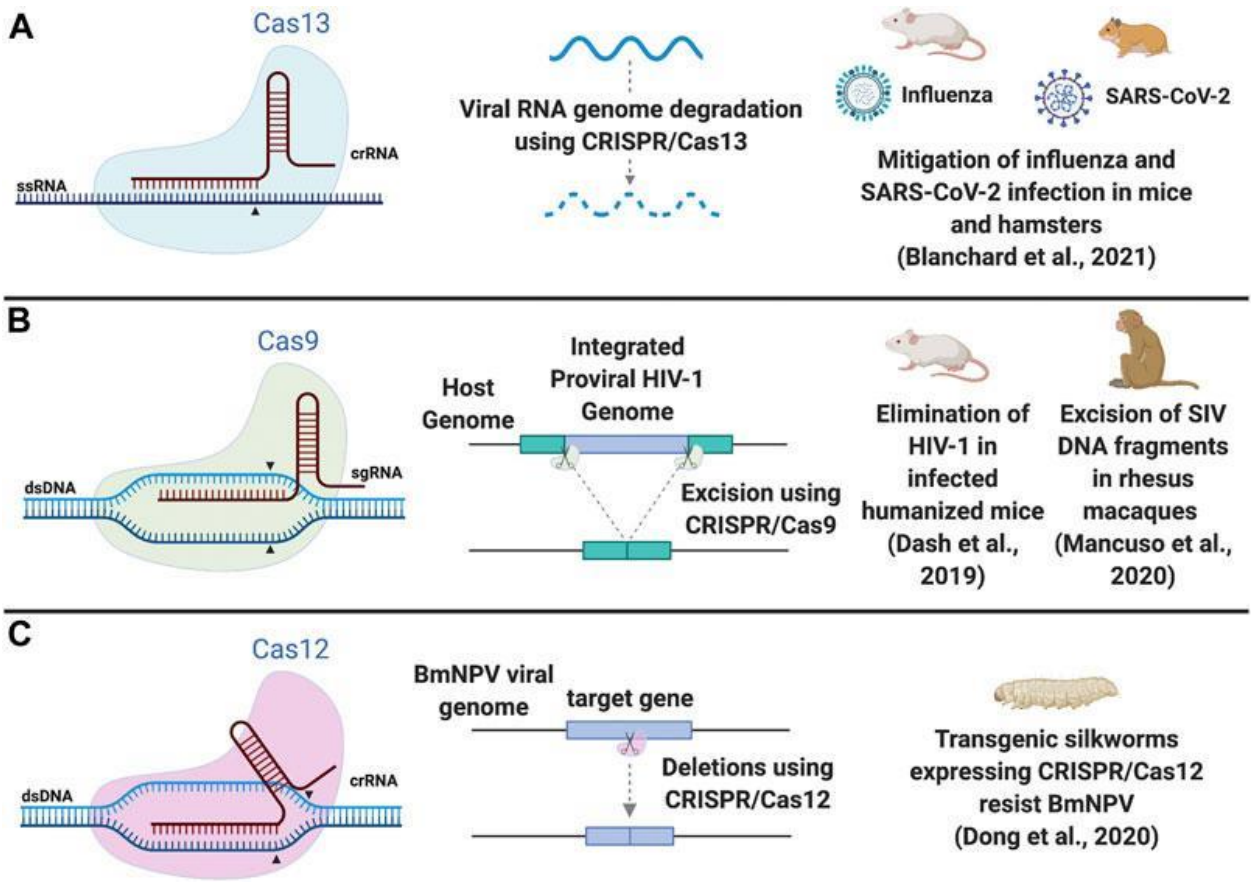

Figure 4 CRISPR-based antiviral mechanisms for RNA and DNA viruses. (A) Cas13 degrades viral ssRNA genomes (e.g., influenza, SARS-CoV-2). (B) Cas9 excises or disrupts integrated viral DNA (e.g., HIV-1) via double-strand cleavage. (C) Cas12 creates staggered DNA cuts to delete targeted regions in DNA viruses (e.g., BmNPV). Adapted from Baddeley & Isalan (2021), CC BY 4.0

Chapter 3

CRISPR in Antiviral Defense

Viruses pose some of the most adaptive and persistent challenges in biology, primarily due to their rapid mutation rates and intimate dependence on host cellular machinery. Traditional antiviral drugs often target viral enzymes or replication intermediates, but resistance quickly emerges as viral populations evolve. The programmable nature of CRISPR systems offers a transformative alternative: instead of relying on fixed molecular targets, CRISPR can be retuned against newly emerging viral sequences within days, making it a potentially agile tool for pandemic response (Chertow, 2018; Abbott et al., 2020).

3.1. Principles of CRISPR-based antiviral strategies

The use of CRISPR for antiviral purposes builds directly upon its native role as a component of the adaptive immune system. In engineered settings, the CRISPR–Cas machinery can be designed to recognize and cleave viral DNA or RNA sequences within infected cells, disrupting replication or permanently inactivating latent genomes (Wang et al., 2019). Depending on the viral genome type, different effectors are selected:

- DNA viruses such as herpesviruses, hepatitis B virus (HBV), and human papillomavirus (HPV) are typically targeted using Cas9 or Cas12 nucleases that induce double-strand breaks in viral episomes or integrated genomes (Kennedy et al., 2014; Ramanan et al., 2015).
- RNA viruses, including influenza, SARS-CoV-2, and HIV, can be countered using Cas13 effectors that degrade viral RNA transcripts or genomes without altering host DNA (Abudayyeh et al., 2016; Freije et al., 2019).

These approaches embody a key conceptual shift: CRISPR acts not as a conventional drug, but as a programmable immune layer, capable of evolving alongside viral diversity.

CRISPR antiviral design generally follows three steps. First, viral genomic regions critical for replication or structural integrity are identified, often through bioinformatic scanning of conserved motifs. Second, single-guide RNAs (sgRNAs or crRNAs) are computationally optimized for on-target binding while minimizing off-target interactions with the host genome. Finally, the CRISPR components are delivered, via plasmids, viral vectors, or nanoparticle

systems, into infected or susceptible cells, where they enact targeted cleavage or degradation of viral nucleic acids (Li et al., 2022).

One of CRISPR's significant advantages is its rapid reconfigurability. During the COVID-19 pandemic, researchers demonstrated that Cas13-based systems could be retargeted to new viral variants within days of sequence release (Abbott et al., 2020). This responsiveness is unmatched by traditional vaccine or small-molecule development pipelines.

3.2. CRISPR applications against DNA viruses

Among the earliest demonstrations of CRISPR's antiviral potential involved DNA viruses, which present relatively stable and accessible genomic targets. The replication of double-stranded DNA viruses often involves episomal intermediates or integrated viral DNA, providing defined substrates for Cas9-mediated cleavage.

A prominent example is the targeting of hepatitis B virus (HBV), a pathogen that establishes persistent infection through the covalently closed circular DNA (cccDNA) reservoir in hepatocyte nuclei. Cas9 systems directed against conserved regions of the HBV surface antigen or polymerase genes have achieved substantial viral suppression in cell and animal models, with some studies reporting over 90% reduction in viral replication (Ramanan et al., 2015; Kostyushev et al., 2021). While complete eradication remains difficult due to the persistence of episomal cccDNA and delivery inefficiencies, these findings highlight the feasibility of functionally silencing chronic viral reservoirs.

Similarly, CRISPR-mediated disruption of human papillomavirus (HPV) oncogenes E6 and E7 has shown potential for reversing malignant transformation in cervical cancer cells. In cultured models, Cas9-induced mutations within these oncogenes restored p53 and Rb tumor suppressor pathways, leading to selective apoptosis of infected cells (Kennedy et al., 2014; Zhen et al., 2014). Such therapeutic mechanisms suggest that CRISPR may complement or replace existing antiviral and anticancer modalities where integration events drive disease.

In herpes simplex virus (HSV) infections, which feature latent DNA reservoirs in neuronal cells, CRISPR has been applied to disrupt immediate early genes essential for viral reactivation. Although complete latency eradication remains elusive, the ability to reduce viral genome copy numbers in vivo marks a substantial advance toward functional cures (van Diemen et al., 2016).

Despite these successes, several challenges remain intrinsic to DNA virus targeting. Viral genomes can mutate near the guide RNA binding site, leading to escape variants that evade

cleavage. Multiplexed CRISPR strategies, using several guides targeting distinct conserved loci, have been shown to delay or prevent escape, though this approach increases vector complexity (Li et al., 2022). Additionally, long-term expression of Cas nucleases in host cells raises concerns regarding genotoxicity and immune activation, necessitating transient or inducible delivery strategies.

3.3. CRISPR against RNA viruses

RNA viruses, due to their high mutation rates and rapid replication speed, pose a more formidable challenge for conventional antiviral design. CRISPR–Cas13, which targets single-stranded RNA, has emerged as a particularly suitable effector for combating these pathogens. Unlike DNA-targeting nucleases, Cas13 does not require a protospacer adjacent motif (PAM); instead, it recognizes a protospacer-flanking site (PFS), which provides broad flexibility in guide design (Abudayyeh et al., 2016).

In 2019, Freije and colleagues introduced PAC-MAN (Prophylactic Antiviral CRISPR in human cells), a Cas13-based system capable of degrading RNA sequences from influenza A and coronavirus (Freije et al., 2019; Abbott et al., 2020). By designing crRNAs against highly conserved regions of the viral genome, the system suppressed viral replication across multiple strains in vitro. This study established a template for pandemic preparedness, enabling the rapid deployment of predesigned CRISPR libraries against emerging RNA viruses.

Beyond influenza and coronaviruses, CRISPR–Cas13 has been tested against dengue, Zika, and enteroviruses, demonstrating potent antiviral activity in mammalian and mosquito models (Metsky et al., 2022). Its transient activity and RNA-targeting nature reduce the risk of permanent genome alterations, making Cas13-based approaches comparatively safer for somatic applications.

Nevertheless, the extraordinary mutation rates of RNA viruses can still lead to target mismatches that compromise CRISPR efficacy. To counter this, several research groups have developed multiplexed Cas13 strategies, employing pools of crRNAs that collectively cover conserved genomic regions, thereby limiting the potential for viral escape (Li et al., 2023). Moreover, delivery remains a critical obstacle, particularly in tissues with high infection loads such as the lungs. Lipid nanoparticles, viral vectors, and exosome-mediated transport are under active evaluation as delivery platforms for Cas13 ribonucleoproteins or mRNAs (Zhang et al., 2023).

An additional consideration lies in collateral activity: once activated, Cas13 can indiscriminately cleave surrounding RNA molecules, a property harnessed in diagnostics but problematic in therapeutics. Engineering efforts have focused on attenuating or temporally restricting collateral cleavage to minimize cytotoxicity while retaining antiviral efficacy (Knott & Doudna, 2018).

3.4. Delivery strategies and translational challenges

While in vitro success has established CRISPR's antiviral promise, clinical translation depends largely on delivery efficiency, target specificity, and immune compatibility. Unlike small molecules or antibodies that diffuse passively, CRISPR systems require the transport of large macromolecular complexes, Cas proteins, guide RNAs, or their genetic templates, into target cells where viruses are replicated. Each delivery platform offers distinct trade-offs between precision, duration, and safety.

Viral vectors, such as adeno-associated virus (AAV) and lentivirus, offer high transduction efficiency but have limited cargo capacity and a potential for insertional mutagenesis. The relatively large size of Cas9 (~4.2 kb) often exceeds the packaging limits of standard AAVs, prompting the use of smaller orthologs such as *Staphylococcus aureus* Cas9 (SaCas9) or split-Cas systems that reassemble within host cells (Ran et al., 2015). Lentiviral vectors, although capable of accommodating larger inserts, integrate into the host genome, which raises biosafety concerns, particularly for transient antiviral interventions.

Non-viral systems have gained traction for their flexibility and improved safety. Lipid nanoparticles (LNPs) can encapsulate Cas mRNA and guide RNAs for transient cytoplasmic expression, a strategy that achieved clinical success in transthyretin amyloidosis (Gillmore et al., 2021). The same principle could be applied to antiviral CRISPR therapies targeting hepatotropic or respiratory viruses, where localized delivery could enhance concentration and reduce systemic exposure.

Emerging physical delivery methods, such as electroporation, microinjection, and nanoneedles, offer high efficiency in controlled laboratory settings but remain impractical for systemic viral infections. Alternatively, biological carriers, including engineered bacteriophages or extracellular vesicles, have been explored for the selective delivery of CRISPR cargo to infected cells (Lino et al., 2018). These vehicles mimic natural tropism, enhance tissue specificity, and reduce immune clearance.

Despite these advances, achieving uniform and safe distribution across infected tissues remains a formidable challenge. Many viruses establish reservoirs in immune-privileged or anatomically sequestered sites, such as neuronal ganglia in HSV or hepatocyte nuclei in HBV, where delivery vectors rarely penetrate effectively. Furthermore, pre-existing immunity to Cas proteins derived from bacterial species (e.g., *Streptococcus pyogenes*) can neutralize Cas nucleases or trigger an inflammatory response (Charlesworth et al., 2019). Current research aims to address these barriers by developing orthologous Cas effectors from non-pathogenic microbes and by employing immune shielding strategies utilizing biomimetic nanoparticles or transient RNA-based delivery.

3.5. Resistance, mutational escape, and multiplexing strategies

One of the most critical limitations of CRISPR antiviral systems is the emergence of viral escape mutants. Since CRISPR relies on precise sequence complementarity between the guide RNA and target genome, even single-nucleotide polymorphisms can abrogate binding or cleavage, restoring viral replication (Wang et al., 2019). This phenomenon mirrors drug resistance in classical antivirals but occurs through genetic drift rather than enzymatic modification.

To counteract this, researchers have developed multiplexed CRISPR designs, in which several guide RNAs simultaneously target distinct conserved loci across essential viral genes. By diversifying the attack sites, the likelihood of simultaneous escape mutations becomes statistically negligible (Li et al., 2023). For example, in the PAC-MAN framework, multiplexed Cas13 crRNAs effectively suppressed diverse coronavirus strains, including SARS-CoV-2 variants, without detectable viral rebound in vitro (Abbott et al., 2020).

Another promising approach involves targeting host dependency factors, such as genes or pathways that viruses hijack for replication but are nonessential to the host. By using CRISPR to transiently knock down or repress such host factors, broad-spectrum antiviral effects can be achieved without direct viral genome editing. This strategy was validated in cell-based studies where CRISPR interference of *CCR5* or *CXCR4* reduced HIV infection (Kaminski et al., 2016). However, the challenge lies in balancing antiviral efficacy with cellular viability, as host-targeted approaches risk unintended physiological disruptions.

Resistance can also be mitigated through iterative guide redesign, where viral genomic surveillance informs real-time CRISPR retargeting. This feedback loop resembles adaptive

immunity at the population level, potentially forming the basis of "smart" antiviral systems capable of evolving in response to viral evolution (Esvelt, 2023).

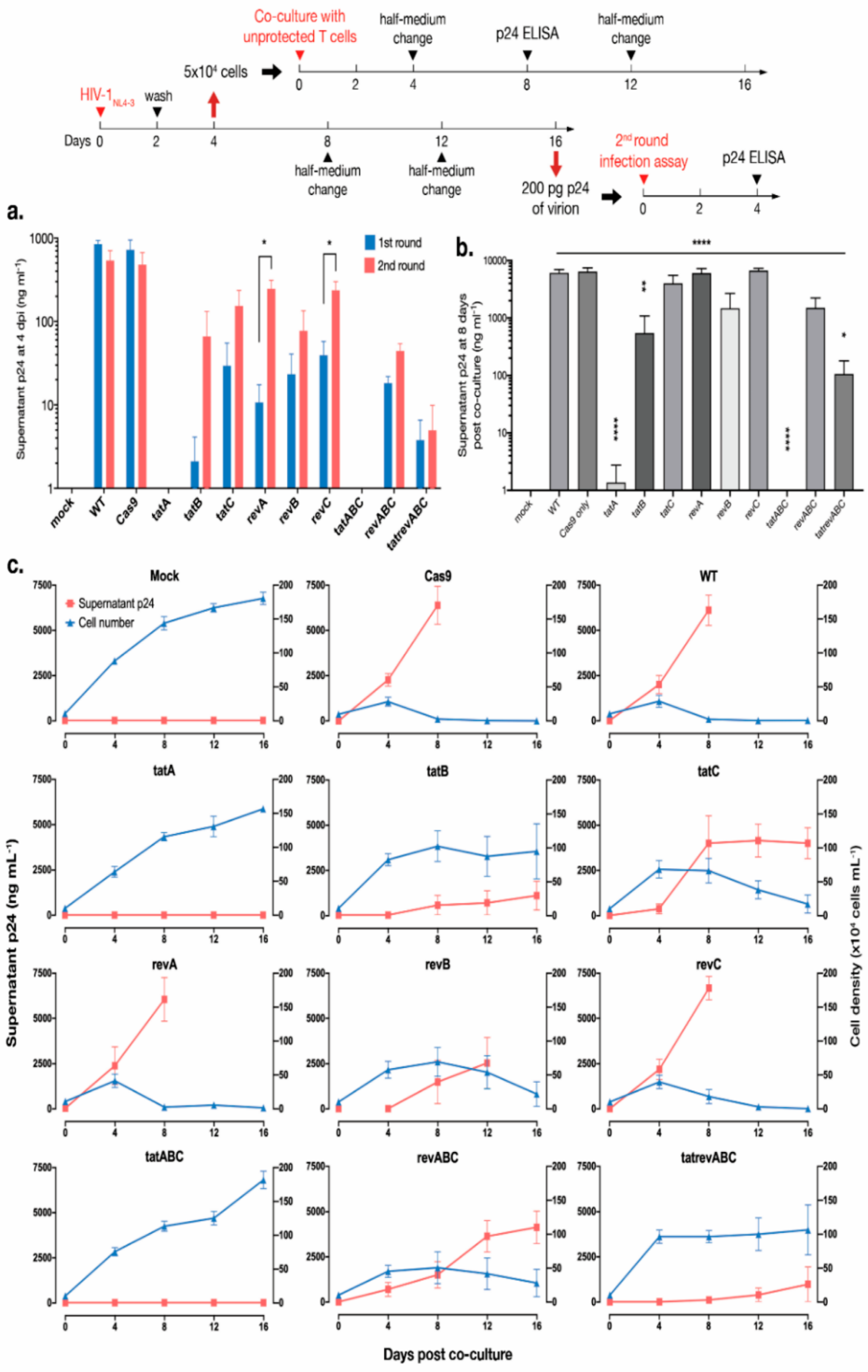

Figure 5 Multiplexed CRISPR–Cas9 limits viral escape compared with single-guide designs. Panel (a) shows replication metrics across first vs. second infection rounds; panels (b–c) show co-culture assays demonstrating sustained suppression for multiplexed tat targeting (tatABC) versus rebound with some single-guide or rev-only designs. Adapted from Ophinni et al. (2020), CC BY 4.0

3.6. Clinical translation and ethical considerations

The path from experimental antiviral design to clinical implementation is complex, as CRISPR interventions blur traditional distinctions between gene therapy, antiviral pharmacology, and immune engineering. Regulatory agencies classify most CRISPR-based antiviral constructs as gene therapy products, subjecting them to rigorous evaluation for biosafety, biodistribution, and genotoxicity (U.S. Food and Drug Administration [FDA], 2023). This oversight, although essential, has slowed the clinical translation compared to other CRISPR applications in oncology and hematology. However, the conceptual promise remains compelling: programmable antiviral systems could one day serve as platform therapeutics, reconfigurable to address novel viral threats like software updates in digital infrastructure.

Human trials have begun to explore this potential in related contexts. The NTLA-2001 study (Gillmore et al., 2021) demonstrated the safe in vivo delivery of Cas9 to hepatocytes, proving that transient gene editing in humans is feasible. Building upon this precedent, preclinical research is adapting similar lipid-nanoparticle frameworks for antiviral delivery, particularly for hepatotropic viruses such as HBV or HCV. Another avenue involves the ex vivo modification of autologous immune cells, where CRISPR edits confer viral resistance —an approach reminiscent of the CCR5 knockouts developed for HIV (Xu et al., 2019). Such strategies emphasize reversible, cell-specific interventions rather than permanent genomic alterations in somatic tissues.

From an ethical standpoint, antiviral CRISPR therapy raises new questions about equity, consent, and ecological impact. Programmable immunity could easily widen disparities between high-income and low-income regions if access remains limited by manufacturing cost or intellectual-property constraints (O'Neill, 2020). Moreover, the prospect of using gene-editing systems for population-scale viral control, such as self-spreading antiviral vectors, demands stringent oversight to prevent ecological imbalance or accidental recombination with circulating pathogens (Esvelt, 2023). Transparency in design, data sharing, and containment practices will therefore determine whether CRISPR antivirals evolve as instruments of global health or as technologies of unintended risk.

The public-perception dimension further complicates adoption. Gene editing still carries cultural and ethical baggage, often conflated with germline modification debates. Effective communication, clarifying that antiviral CRISPR operates transiently and non-hereditarily, is crucial for public trust and informed consent. Initiatives integrating community bioethics

panels and open-source guide registries may help demystify these interventions and ensure inclusive governance.

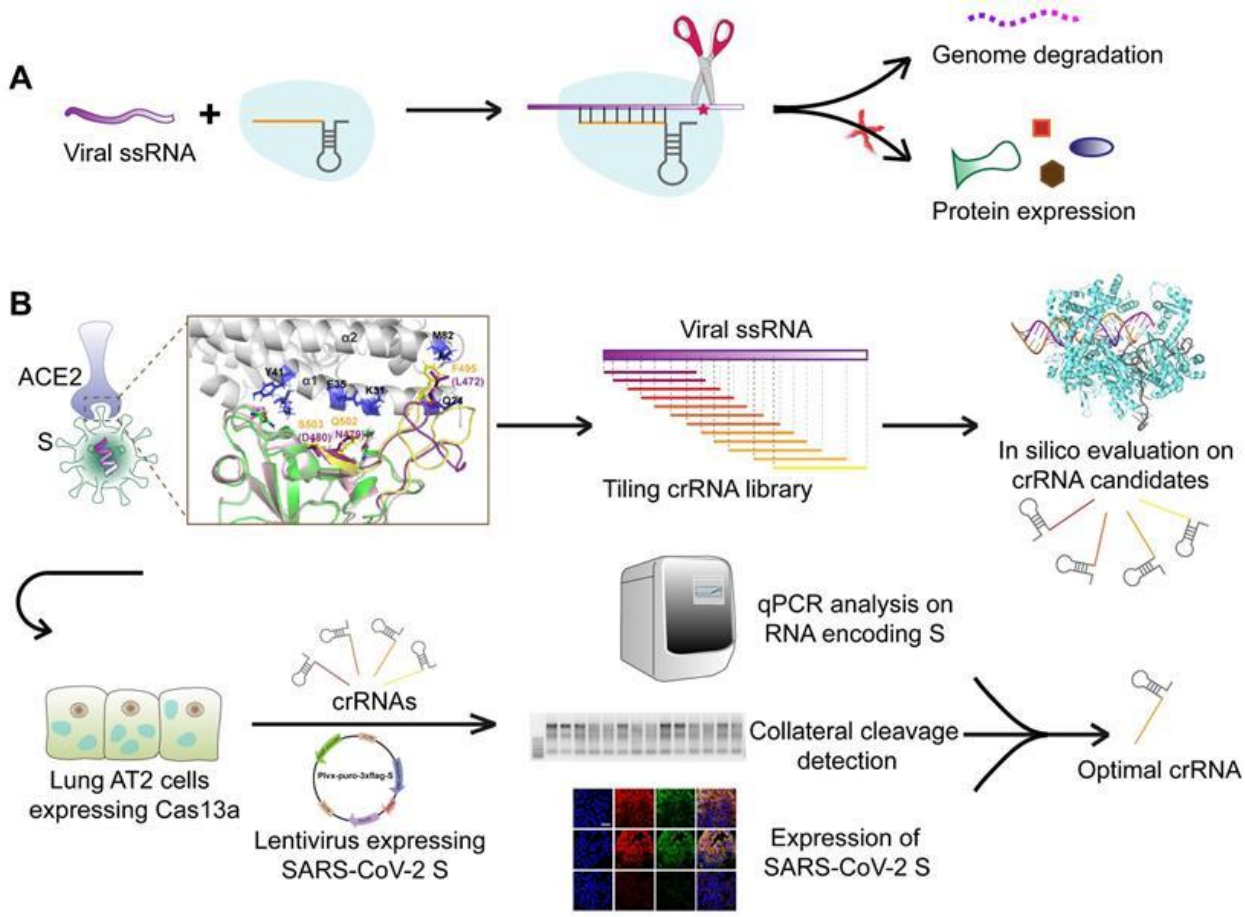

Figure 6 End-to-end workflow for CRISPR antiviral development: target selection and crRNA tiling, in-silico screening, cellular assays (qPCR/protein expression), and optimization to identify lead crRNAs for therapeutic Cas13 deployment. Adapted from Wang et al. (2021), licensed under CC BY 4.0.

3.7. Integrative perspective and outlook

Taken together, CRISPR-based antiviral technologies illustrate how synthetic biology can transform defensive medicine. They offer unprecedented speed in design, modularity across pathogens, and precision unmatched by chemical antivirals. Yet they also expose a paradox at the heart of biotechnology: the closer we come to mastering biological control, the greater our responsibility to wield it wisely. The effectiveness of CRISPR antivirals will ultimately depend not only on biochemical ingenuity but also on delivery innovation, equitable access, and robust ethical frameworks.

Future progress is likely to converge along three complementary axes. First, miniaturized and immune-silent Cas effectors will enhance delivery to difficult tissues. Second, real-time genomic surveillance integrated with automated guide-design algorithms could establish adaptive antiviral pipelines capable of responding within weeks of an outbreak. Third, international biosafety harmonization, defining standards for containment, validation, and data transparency, will be essential for translating laboratory promise into global health resilience.

By enabling programmable immunity, CRISPR systems blur the line between therapy and prevention, giving rise to a new paradigm of molecularly intelligent medicine. As the next sections demonstrate, the same properties that empower CRISPR antivirals, programmability, sequence specificity, and modularity, also underpin its applications against antimicrobial resistance and in next-generation diagnostics.

Chapter 4

CRISPR in Antimicrobial Applications

Antimicrobial resistance (AMR) threatens to nullify decades of medical progress. The World Health Organization projects that drug-resistant infections could cause up to ten million deaths annually by 2050 if current trends persist (WHO, 2022). Conventional antibiotics exert selective pressure that accelerates resistance evolution, whereas CRISPR-based antimicrobials promise a targeted, evolution-conscious approach. By exploiting the precision of nucleic-acid recognition, CRISPR can selectively eliminate pathogenic strains while sparing beneficial microbiota, a paradigm shift from broad-spectrum antibiotics to sequence-specific interventions (Bikard et al., 2014; Citorik et al., 2014).

4.1. Mechanistic rationale

CRISPR antimicrobials operate by introducing double-strand breaks or lethal mutations within essential or resistance-conferring genes of bacteria. When CRISPR components, typically Cas9 or Cas12a with customized guide RNAs, are introduced into bacterial populations, they induce targeted genomic cleavage. Because bacteria generally lack efficient non-homologous end-joining pathways, the resulting double-strand breaks are fatal (Bikard et al., 2014). This mechanism enables strain-specific eradication without perturbing the commensal flora.

A complementary strategy involves targeting mobile genetic elements such as plasmids and integrons that disseminate resistance genes. By targeting CRISPR nucleases against plasmid replication origins or resistance determinants (e.g., blaNDM-1 or mecA), researchers have successfully achieved plasmid curing in multi-drug-resistant *Escherichia coli* and *Staphylococcus aureus* isolates (Citorik et al., 2014; Yosef et al., 2015). This selective plasmid clearance not only restores antibiotic susceptibility but also prevents horizontal transfer of resistance alleles within microbial communities.

Beyond lethality, deactivated Cas variants (dCas9, dCas12) provide transcriptional control over virulence factors. CRISPR interference (CRISPRi) systems have been used to repress genes encoding toxins, adhesion proteins, and quorum-sensing regulators, effectively attenuating pathogenicity without killing the cell (Peters et al., 2019). Such “anti-virulence” approaches reduce evolutionary pressure for resistance emergence, offering a subtler ecological footprint than bactericidal methods.

4.2. Delivery platforms for bacterial targets

The central obstacle to CRISPR-based antimicrobial deployment remains the efficient delivery of CRISPR-based antimicrobial agents into bacterial populations, particularly in complex host environments. Several strategies have been developed, each balancing specificity, payload size, and scalability.

1. Bacteriophage-mediated delivery: Engineered lytic or temperate phages serve as natural vectors for CRISPR cargo. The first proof-of-concept study demonstrated phage-delivered Cas9 that selectively eradicated antibiotic-resistant *S. aureus* while leaving sensitive strains unharmed (Bikard et al., 2014). More recently, synthetic phagemid systems have expanded their host range and enabled the packaging of larger CRISPR constructs, including multiplexed guide arrays. Phage delivery ensures infection specificity but requires continual adaptation to phage-host coevolution and regulatory oversight concerning recombinant phage release.
2. Conjugative plasmids: Mobilizable plasmids exploiting bacterial conjugation machinery can transfer CRISPR modules horizontally across related strains (Rodrigues et al., 2019). This approach is particularly promising for targeting pathogens in mixed microbial consortia or biofilms. However, conjugation is limited by host range and may inadvertently propagate the CRISPR-Cassette if not self-limiting. To mitigate this, “kill-switch” or suicide-plasmid designs incorporate self-deactivation once target clearance is achieved.
3. Nanoparticle and vesicle carriers: Lipid nanoparticles, polymeric nanocarriers, and bacterial outer-membrane vesicles have been adapted for CRISPR cargo encapsulation. These vehicles protect nucleic acids from enzymatic degradation and facilitate uptake by Gram-negative bacteria that are typically resistant to phage infection (Qiu et al., 2022). Surface functionalization with antimicrobial peptides or targeting ligands enhances cell-type specificity, an active area of formulation research.

Each delivery route entails a different risk profile. Phage-based systems require environmental containment and raise ecological concerns about horizontal gene transfer. Plasmid approaches demand precise control to avoid long-term dissemination. Nanoparticles must overcome mucus barriers and immune clearance in vivo. The success of CRISPR antimicrobials will therefore depend on coupling molecular precision with ecological stewardship.

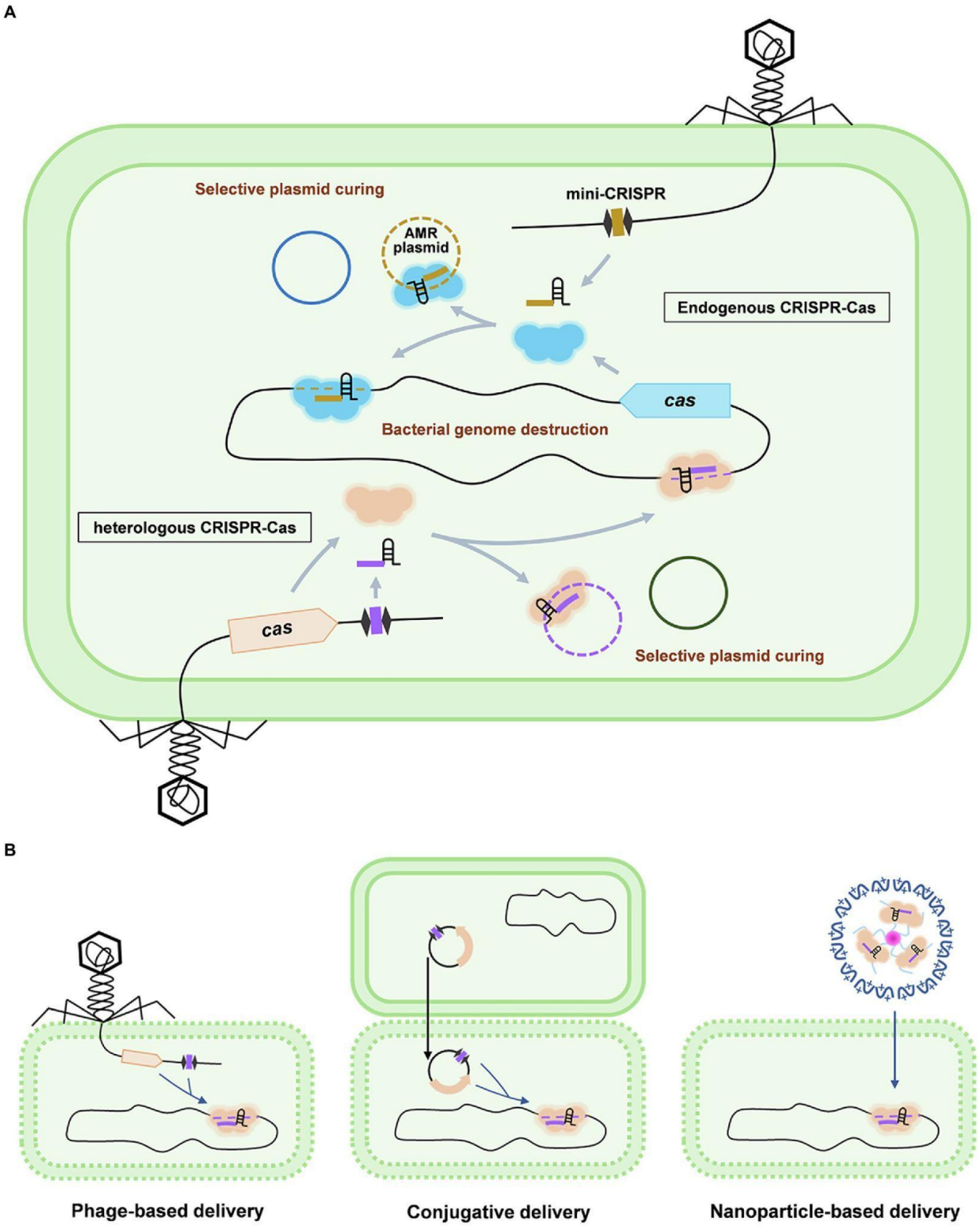

Figure 7 Working mechanisms and delivery strategies for CRISPR-Cas antimicrobials. (A) Endogenous vs. heterologous CRISPR killing and selective plasmid curing. (B) Delivery routes: phage-based, conjugative plasmid-based, and nanoparticle-based vectors. Adapted from Duan et al. (2021), CC BY 4.0

4.3. Applications in drug-resistant pathogens

CRISPR antimicrobials have shown notable progress against several priority bacterial pathogens. Early proof-of-concept studies targeted *Staphylococcus aureus*, *Escherichia coli*, and *Clostridioides difficile*, demonstrating that Cas9-mediated cleavage of resistance determinants could restore antibiotic sensitivity. In *S. aureus*, phage-delivered CRISPR constructs eliminated strains harboring the *mecA* gene, which is responsible for methicillin resistance, while leaving commensal populations intact (Bikard et al., 2014). Similarly, CRISPR plasmids targeting blaNDM-1 and blaCTX-M successfully eradicated extended-spectrum β-lactamase (ESBL) plasmids in multidrug-resistant E. coli, thereby reversing resistance to carbapenems and cephalosporins (Yosef et al., 2015).

In *Enterococcus faecalis*, a high-risk nosocomial pathogen, conjugative CRISPR systems were engineered to eliminate vancomycin-resistance genes (vanA and vanB) selectively. When deployed in murine gut models, these conjugative plasmids reduced colonization by resistant strains without disturbing indigenous commensals (Rodrigues et al., 2019). This precision reflects the ecological advantage of CRISPR antimicrobials, targeting only pathogenic genotypes while preserving microbiome balance.

The versatility of CRISPR also extends to Gram-negative pathogens such as *Pseudomonas aeruginosa*, where CRISPRi systems have been used to silence efflux-pump regulators and quorum-sensing pathways, thereby resensitizing bacteria to conventional antibiotics (Peters et al., 2019). These combinatorial strategies suggest that CRISPR may complement existing antibiotic regimens rather than replace them, acting as an adjuvant that weakens resistance barriers.

Recent work in tuberculosis models demonstrates further promise. Mycobacteria are traditionally resistant to gene delivery, but optimized phagemid systems carrying Cas12a have achieved targeted disruption of *inhA* and *katG*, key genes involved in isoniazid resistance (Yan et al., 2023). Although still in preclinical stages, these results indicate that CRISPR could eventually address one of the most intractable global AMR crises.

Table 2 CRISPR Antimicrobial Strategies Targeting Drug-Resistant Bacteria

Pathogen / Condition	Genetic Target	Cas System	Delivery Method	Outcome
MRSA (Staphylococcus aureus)	*mecA* (β-lactam resistance gene)	Cas9	Engineered bacteriophage	Selective killing of resistant strains while sparing commensals
ESBL-producing *E. coli*	*blaNDM-1*, *blaCTX-M* (carbapenem / cephalosporin resistance)	Cas9	Conjugative plasmid	Plasmid curing restores antibiotic susceptibility
Vancomycin-resistant Enterococcus (VRE)	*vanA / vanB* (cell-wall modification genes)	Cas9	Self-transmissible CRISid plasmid	Depletion of resistant subpopulation in gut model
Pseudomonas aeruginosa (biofilm-forming)	*lasR* (quorum-sensing regulator)	dCas9 (CRISPRi)	Plasmid / nanoparticle	Biofilm disruption and resensitization to antibiotics
Mycobacterium tuberculosis	*inhA*, *katG* (isoniazid resistance genes)	Cas12a	Optimized phagemid	Reduced viability of drug-resistant mycobacteria in vitro
Clostridioides difficile	Toxin genes (*tcdA/tcdB*)	Cas9	Phage-like delivery particles	Toxin repression and reduced cytotoxicity in host cells

4.4. Biofilm control and microbiome-level interventions

Biofilms present a distinct challenge in infectious disease management because their extracellular matrix and metabolic heterogeneity protect resident bacteria from antibiotics and host immunity. CRISPR-based antimicrobials offer new strategies for disrupting biofilm architecture at the genetic level. Targeting quorum-sensing genes, such as luxS and lasR, has been shown to inhibit biofilm maturation and virulence in P. aeruginosa and Streptococcus mutans, leading to the dismantling of mature biofilms and improved antibiotic penetration (Zuberi et al., 2021).

Another innovative approach employs programmable dCas9 repressors to modulate community composition within polymicrobial biofilms. By selectively silencing adhesion or exopolysaccharide genes in dominant pathogens, CRISPRi systems can shift the ecological balance toward nonpathogenic species, restoring microbiome homeostasis (Peters et al., 2019). This concept, sometimes referred to as precision microbiome editing, represents a transition from eradication to ecological rehabilitation, a strategy increasingly valued in chronic infections, such as cystic fibrosis or periodontitis.

Delivery of CRISPR components within biofilms remains a technically demanding task. Engineered bacteriophages capable of penetrating biofilm matrices or nanoparticles conjugated with enzymes, such as DNase I, to degrade extracellular DNA have improved access to embedded cells (Qiu et al., 2022). Combining these carriers with CRISPR payloads may finally overcome one of the most persistent obstacles in antimicrobial therapy.

4.5. Safety, regulation, and translational outlook

The translation of CRISPR antimicrobials from laboratory systems to clinical or environmental use raises unique biosafety and governance questions. Because these constructs can self-propagate through microbial populations, their deployment must follow strict containment and monitoring protocols comparable to those used for genetically modified organisms (Esvelt, 2023). Regulatory frameworks are still evolving; most agencies classify phage-delivered CRISPR constructs as “biological control agents,” which requires an assessment of horizontal gene transfer risks and ecological persistence before field release.

Therapeutically, immunogenicity is less problematic than in eukaryotic applications, yet unintended gene flow or off-target effects in commensal bacteria remain concerns. Incorporating self-destruct modules, such as genetic circuits that deactivate the CRISPR-Cas9 after target engagement, can enhance biosafety (Crawford et al., 2021). Transparent data reporting and public engagement are equally important for social acceptance, particularly when interventions may impact shared environments, such as wastewater systems or agricultural settings.

Looking forward, the most promising trajectory involves hybrid therapeutics that combine CRISPR specificity with traditional antibiotics or bacteriophage therapy. By weakening resistance determinants genetically and then applying chemical or biological agents, synergistic clearance can be achieved at lower drug concentrations. Advances in microfluidic screening,

AI-based guide-RNA design, and synthetic-biology containment will continue to refine these platforms.

CRISPR antimicrobials mark a conceptual shift from reactive treatment to genomic-level infection management, aligning molecular precision with ecological mindfulness. Their successful integration into medicine and public health will depend on maintaining this balance, leveraging specificity without compromising biosafety or microbial diversity.

Table 3 Representative CRISPR-based antimicrobial applications targeting drug-resistant bacteria, showing genetic targets, CRISPR effectors, delivery routes, and experimental outcomes

Pathogen / Context	**Resistance or Virulence Target**	**Cas System Used**	**Delivery Method**	**Experimental Outcome**
MRSA (Staphylococcus aureus)	*mecA* (β-lactam resistance gene)	Cas9	Engineered phage	Selective killing of resistant strains, spares commensals
ESBL-producing *E. coli*	*blaNDM-1* / *blaCTX-M* (carbapenem & cephalosporin resistance)	Cas9	Conjugative plasmid	Plasmid curing restores antibiotic susceptibility
Vancomycin-resistant Enterococcus (VRE)	*vanA / vanB*	Cas9	Self-transmissible CRISPR plasmid	Depletion of resistant cells in gut microbiome model
Pseudomonas aeruginosa (biofilms)	*lasR* (quorum-sensing regulator)	dCas9 (CRISPRi)	Plasmid / nanoparticle	Biofilm disruption + resensitization to antibiotics
Mycobacterium tuberculosis	*inhA* / *katG* (isoniazid resistance genes)	Cas12a	Phagemid-based delivery	Reduction in survival of drug-resistant MTB in vitro
Clostridioides difficile	*tcdA / tcdB* (toxin genes)	Cas9	Phage-like delivery particles	Toxin suppression + protection of host cells

Chapter 5

CRISPR-Based Diagnostics

CRISPR diagnostics convert nucleic-acid recognition into an analytic signal by exploiting the target-activated “collateral” nuclease activity of certain Cas effectors. Cas12, upon binding a cognate double-stranded DNA target, indiscriminately cleaves nearby single-stranded DNA reporters; Cas13 does the same to single-stranded RNA reporters after recognizing an RNA target (Chen et al., 2018; Abudayyeh et al., 2016). These biochemical properties enabled the development of rapid, highly specific assays that rival or complement RT-PCR, offering simpler hardware requirements and faster assay cycles. Since 2017, the CRISPR diagnostics landscape has expanded from first-generation platforms such as SHERLOCK and DETECTR to integrated, field-ready formats that reduce hands-on time, minimize contamination, and support multiplexed surveillance (Gootenberg et al., 2017; Broughton et al., 2020).

5.1. Mechanistic principles and platform lineage

Early demonstrations of SHERLOCK (Specific High-sensitivity Enzymatic Reporter unLOCKing) showed that Cas13a, combined with recombinase polymerase amplification (RPA) or T7 transcription, could detect attomolar quantities of viral RNA with fluorescent or lateral-flow readouts (Gootenberg et al., 2017; Gootenberg et al., 2018). In parallel, DETECTR leveraged Cas12a to sense DNA amplicons generated by loop-mediated isothermal amplification (LAMP) or RPA, enabling one-hour workflows for pathogens such as human papillomavirus and, later, SARS-CoV-2 (Chen et al., 2018; Broughton et al., 2020). These foundational studies established the now-standard components of CRISPR diagnostics: a rapid amplification step to boost template copies; a CRISPR detection mix with guide RNA and Cas enzyme; and a universal reporter that produces a fluorescent signal or a visible line on a lateral-flow strip when cleaved.

Second-generation platforms focused on integration and scale. CARMEN combined thousands of Cas13 reactions in a microarray of nanoliter droplets to enable high-throughput, multiplexed detection of many viruses in parallel, at once demonstrating CRISPR’s utility for surveillance and underscoring the importance of miniaturization and barcoding for cost control (Ackerman et al., 2020). SHINE condensed amplification, detection, and readout into a single tube with lyophilized reagents, improving compatibility with resource-limited settings and cutting total turnaround to under an hour while maintaining sensitivity comparable to RT-PCR in clinical

samples (Arizti-Sanz et al., 2020). STOPCovid/DETECTR-style one-pot chemistries further simplified workflows by merging RT-LAMP and Cas12 detection at a single temperature, reducing tube-opening steps that otherwise risk amplicon contamination (Joung et al., 2020; Broughton et al., 2020).

A parallel line of development pursued amplification-free detection to avoid biases and contamination introduced by pre-amplification. Using optimized Cas13 kinetics, engineered reporters, and smartphone-based optics, investigators achieved direct detection of SARS-CoV-2 RNA at clinically relevant concentrations in patient samples, albeit with longer acquisition times or specialized optics to maintain sensitivity (Fozouni et al., 2021). While amplification-free assays are not yet as broadly sensitive as amplification-assisted formats, they illustrate a path toward quantitative CRISPR diagnostics with simplified reagent stacks.

5.2. Assay workflow: sample-to-answer considerations

Most CRISPR diagnostic pipelines begin with sample lysis and nucleic-acid extraction or stabilization, proceed through amplification (RPA or RT-LAMP are common), and culminate in a detection phase where Cas12 or Cas13 cleaves a reporter to generate signal. Extraction-free approaches have proven effective for certain matrices, such as saliva or anterior-nares swabs, when heat or chemical lysis buffers are combined with inhibitor-tolerant RT-LAMP, significantly shortening the workflow without catastrophic sensitivity loss (Broughton et al., 2020; Arizti-Sanz et al., 2020). Lyophilization of the amplification and detection mixes improves cold-chain independence and extends shelf life, which is crucial for decentralized testing and low-resource environments (Joung et al., 2020).

Readout choice drives instrumentation needs and scalability. Fluorescent detection enables quantitative or semi-quantitative interpretation and easy integration with microplate readers, but lateral-flow strips support electricity-free deployment and visual interpretation by non-specialists. Electrochemical CRISPR sensors using graphene or gold electrodes have emerged as an attractive middle ground, offering portable instrumentation with high sensitivity and inherent digitization for connectivity to laboratory information systems (Hajian et al., 2019). Regardless of modality, modern CRISPR assays emphasize “closed-tube” architectures to reduce amplicon aerosolization, an operational detail that substantially decreases false positives in routine use.

5.3. Performance metrics and clinical validation

Analytical sensitivity in CRISPR assays typically falls in the range of 10–100 copies per reaction for amplification-assisted formats, with some SHERLOCK/DETECTR variants achieving single-digit copies under optimized conditions (Gootenberg et al., 2018; Broughton et al., 2020). Clinical evaluations compare assay results to an authorized RT-PCR comparator and report positive percent agreement (PPA) and negative percent agreement (NPA), often exceeding 90% in prospective studies when sample handling and pre-analytics are controlled (Broughton et al., 2020; Arizti-Sanz et al., 2020). Time-to-result commonly ranges from 30 to 60 minutes, depending on whether extraction is performed and whether the chemistry is one-pot or two-step.

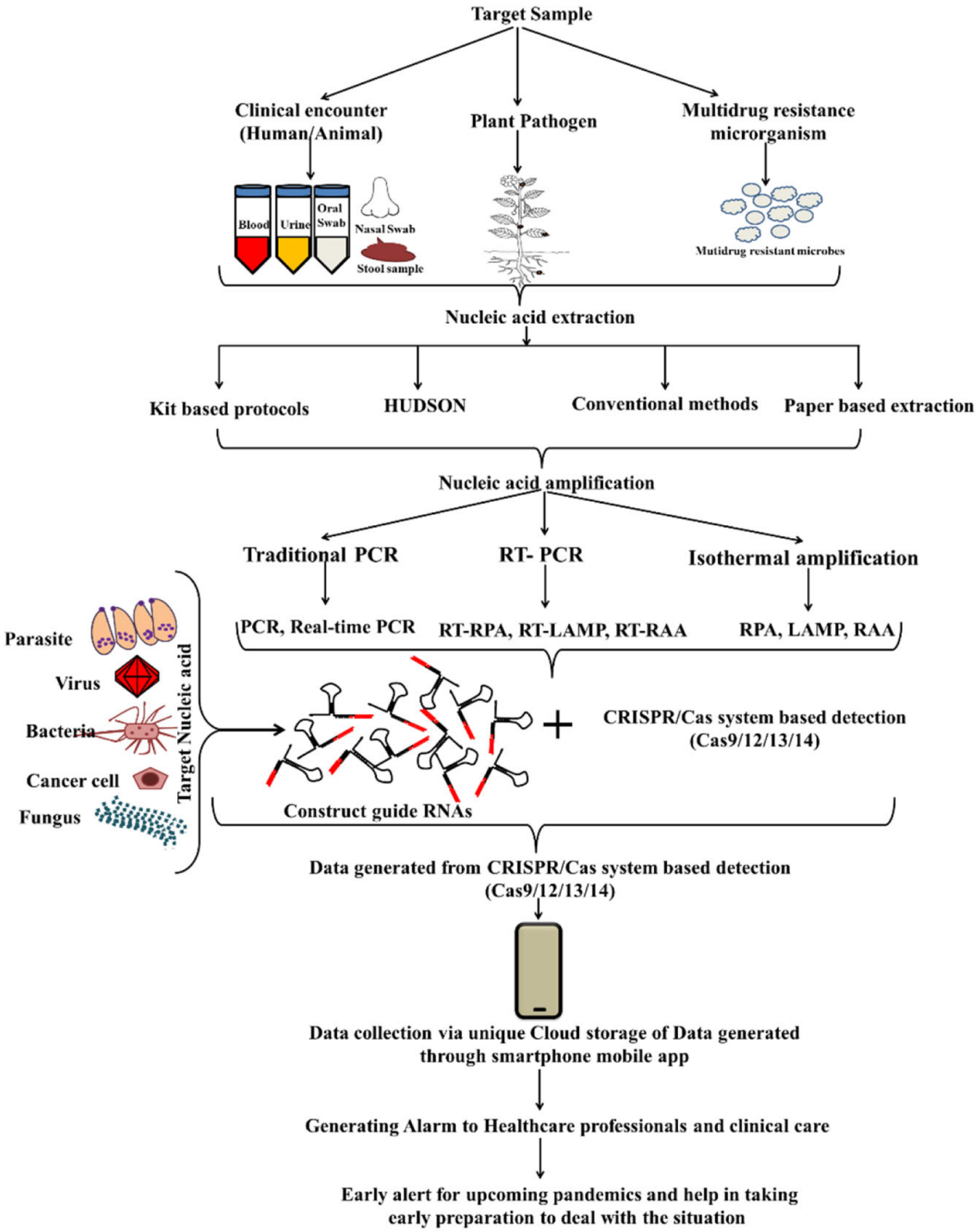

Figure 8 End-to-end CRISPR diagnostic workflow from sample type and nucleic-acid extraction (HUDSON/kit/conventional) through amplification (PCR, RT-PCR, RPA/LAMP/RAA) to CRISPR-Cas detection (Cas9/12/13/14) and result reporting. Adapted from Bhardwaj et al. (2022), licensed under CC BY 4.0

Regulatory precedent now exists for CRISPR diagnostics. During the COVID-19 pandemic, the Sherlock CRISPR SARS-CoV-2 kit obtained U.S. FDA Emergency Use Authorization, followed by a Cas12-based DETECTR BOOST kit that validated an integrated, high-throughput workflow (U.S. Food and Drug Administration, 2020; U.S. Food and Drug Administration, 2022). These authorizations catalyzed broader clinical adoption and provided clear expectations for documentation of inclusivity/exclusivity panels, cross-reactivity testing, and lot-to-lot reproducibility, requirements that have since informed evaluation of non-respiratory targets such as HPV genotyping and antimicrobial-resistance markers.

Table 4 Representative CRISPR-based diagnostic platforms and their core functional characteristics

Platform	**Cas Effector**	**Amplification Method**	**Readout Format**	**Time to Result**	**Sensitivity (Typical LOD)**	**Primary Application**
SHERLOCK	Cas13a	RPA + T7 transcription	Fluorescence or lateral flow strip	~60 min	Attomolar (10^{-18} M)	Viral RNA detection (e.g., Zika, SARS-CoV-2)
DETECTR	Cas12a	RT-LAMP or RPA	Fluorescence or lateral flow strip	30–45 min	fg-level DNA/RNA	DNA virus and HPV screening, COVID-19
STOPCovid / DETECTR BOOST	Cas12b / Cas12a	One-pot RT-LAMP	Lateral flow or plate reader	45–55 min	~100 copies/reaction	SARS-CoV-2 screening (EUA-approved versions)
SHINE	Cas13	Single-tube isothermal workflow	Portable visual or fluorimetric detection	<60 min	Comparable to RT-PCR	Field-deployable viral diagnostics

CARMEN	Cas13	Microdroplet barcoded array	Multiplex fluorescence imaging	Batch-based	Hundreds of targets per run	Large-scale pathogen surveillance
Amplification-free Cas13 systems	Cas13	None (direct detection)	Smartphone or electrochemical readout	20–90 min	~100–200 copies/µL (improving)	Decentralized / point-of-care RNA detection

5.4. Contamination control and quality assurance

Because CRISPR diagnostics frequently involve amplification, preventing carry-over contamination is critical for reliability. The simplest safeguards are closed-tube reactions, combining amplification and detection without reopening the vessel, and uracil-DNA-glycosylase (UDG) treatment, which enzymatically degrades residual amplicons from previous runs (Joung et al., 2020). Many one-pot systems now integrate these controls automatically. Internal amplification controls (IACs) are also recommended to detect inhibition or reagent failure, improving interpretability for field operators (Arizti-Sanz et al., 2020). Batch-level verification typically includes positive and negative controls plus an internal process control for extraction efficiency. These measures align CRISPR diagnostics with ISO 15189 and CLSI molecular testing standards, positioning them for clinical accreditation.

5.5. Multiplexing, variant tracking, and surveillance integration

The programmable nature of CRISPR allows simultaneous detection of multiple genetic targets using distinct guide RNAs or orthogonal enzymes. CARMEN demonstrated hundreds of Cas13 reactions in parallel, providing scalable pathogen panels for respiratory viruses (Ackerman et al., 2020). More recent multiplexed assays employ color-coded fluorophores or spatial separation on paper-based microfluidics to differentiate signals in a single cartridge (Li et al., 2022).

These capabilities extend naturally to variant identification and mutation tracking. For instance, SARS-CoV-2 spike mutations, such as N501Y and E484K, were rapidly incorporated into DETECTR and SHERLOCK assays to discriminate variants of concern (Patchsung et al., 2021). Because guides can be redesigned within hours of new sequence disclosure, CRISPR platforms are increasingly viewed as agile complements to sequencing for surveillance in

decentralized laboratories. Coupling with cloud-based data aggregation further enables real-time epidemiological mapping and decision support.

5.6. Deployment, manufacturing, and cost considerations

Field deployment requires balancing sensitivity with affordability and operational simplicity. Lyophilized reagent kits, stable at ambient temperatures for several months, have brought per-test costs down to the US $3–5 range for lateral-flow formats (Joung et al., 2020). Integration into microfluidic cartridges reduces reagent volumes, minimizes human error, and enables high-throughput testing in resource-limited clinics.

Manufacturing scale-up has also improved quality control. Commercial CRISPR diagnostic kits now undergo standard lot release testing analogous to RT-PCR reagents, with reference materials from the National Institute of Standards and Technology (NIST) or equivalent bodies for calibration. These practices reinforce reproducibility across production batches, a prerequisite for sustainable adoption beyond emergency-use contexts.

5.7. Limitations and continuing challenges

Despite its promise, CRISPR diagnostics face several operational barriers. Quantitation remains semi-qualitative in most lateral-flow implementations, constraining clinical interpretation for viral-load monitoring. Sample matrix effects, such as inhibitors in blood or sputum, can reduce amplification efficiency, necessitating optimized buffer chemistries for each specimen type. In addition, collateral cleavage saturation limits dynamic range, complicating multiplex quantitation without microfluidic compartmentalization.

From a policy perspective, regulatory pathways remain fragmented. While the U.S. FDA has published EUA precedents, standardized guidance for full clearance or for use in non-clinical surveillance is still evolving (U.S. Food and Drug Administration, 2022). Harmonization across jurisdictions will be vital for integrating CRISPR diagnostics into existing molecular-testing infrastructures and for cross-border data exchange.

5.8. Outlook

The coming generation of CRISPR diagnostics will likely merge amplification-free sensing, microfluidic integration, and digital quantitation into cohesive, portable devices. Cas enzymes with improved turnover, such as Cas14 or engineered hyperactive Cas12b, promise faster reaction kinetics at ambient temperature, further reducing equipment demands (Pausch et al.,

2020). Artificial-intelligence-assisted guide-design pipelines are already shortening assay-development timeframes and improving mismatch tolerance prediction.

As the field matures, CRISPR diagnostics are poised to shift from emergency tools to routine, decentralized surveillance platforms, supporting not only infectious disease management but also food safety, agriculture, and environmental monitoring. Their combination of sensitivity, portability, and modularity establishes CRISPR as the most versatile nucleic acid detection technology since PCR.

Chapter 6

Challenges and Limitations

While CRISPR–Cas systems have revolutionized genetic engineering, diagnostics, and pathogen control, their translation into real-world biomedical and biosecurity applications remains constrained by several scientific, technical, and ethical barriers. Understanding these limitations is essential for responsible innovation, as each challenge influences the reliability, safety, and societal acceptance of CRISPR-based technologies (Esvelt, 2023; Koonin & Makarova, 2022).

Table 5 Delivery systems used for CRISPR-based therapeutics and antimicrobial applications, highlighting cargo format, strengths, limitations, and representative use cases.

Delivery Method	Cargo Type(s) Carried	Key Advantages	Major Limitations	Representative Application
AAV (Adeno-associated virus)	Cas DNA + gRNA cassette	Long-term expression, tissue tropism options	Small cargo limit (~4.7 kb), pre-existing immunity	In vivo Cas9 editing (TTR amyloidosis, retinal disease)
Lipid nanoparticles (LNPs)	Cas mRNA + gRNA, or RNP	Transient expression, clinically validated, scalable	Strong liver tropism, limited extrahepatic delivery	NTLA-2001 (first in-human CRISPR therapy), Cas13 antivirals
Engineered bacteriophages	Cas9 plasmid or RNP	Natural bacterial targeting, high specificity	Narrow host range, environmental biosafety concerns	MRSA mecA knockout, phage-delivered anti-AMR systems
Conjugative plasmids	Cas cassette (DNA)	Self-spreading within microbial communities	Horizontal gene transfer risks	VanA/VRE plasmid curing in gut microbiome
Exosomes / Extracellular vesicles (EVs)	Cas RNP	Low immunogenicity, crosses some biological barriers	Manufacturing + loading challenges	Preclinical antiviral and cancer CRISPR delivery
Polymeric or inorganic nanoparticles	Cas RNP or DNA	Tunable charge, stability, surface functionalization	Lower transfection efficiency vs LNP	Experimental antimicrobial and gene-regulation systems

Physical methods (electroporation, microinjection, nanoneedles)	RNP, DNA, or mRNA	High efficiency in ex vivo or local contexts	Not scalable in vivo, tissue damage risks	Ex vivo T-cell engineering, localized antiviral delivery

6.1. Specificity and off-target effects

Off-target activity remains one of the most scrutinized issues in CRISPR implementation. Although guide RNAs are designed to pair specifically with target sequences, mismatches in the seed region or flexible PAM recognition can cause unintended cleavage at homologous genomic sites (Tsai & Joung, 2016). In therapeutic settings, even a small fraction of off-target edits can lead to oncogenic transformations or other unpredictable phenotypes. High-fidelity Cas9 variants such as eSpCas9, SpCas9-HF1, HypaCas9, and Sniper-Cas9 have been engineered to mitigate these effects, each introducing structural mutations that tighten target verification before cleavage (Slaymaker et al., 2016; Chen et al., 2017).

In addition to enzyme engineering, empirical mapping techniques like GUIDE-seq, Digenome-seq, and CIRCLE-seq provide experimental validation of specificity across cell types and genomic contexts (Kim et al., 2015). Integrating these datasets with machine-learning prediction models has improved computational guide design, enabling dynamic risk scoring for off-target sites. Nevertheless, no predictive algorithm guarantees universal accuracy, especially in highly repetitive genomes or when guides overlap with structural variants. For clinical translation, regulatory agencies now emphasize empirical validation of specificity in the exact cell type or tissue where CRISPR will be deployed, rather than relying solely on in silico predictions (U.S. Food and Drug Administration, 2023).

The challenge extends to diagnostics as well. Cas12 and Cas13 effectors exhibit collateral cleavage, which, while advantageous for signal amplification, can reduce signal fidelity if multiple reporter species or background nucleic acids compete in solution (Chen et al., 2018). Balancing this dual nature, specific recognition and promiscuous cleavage, remains a central engineering priority.

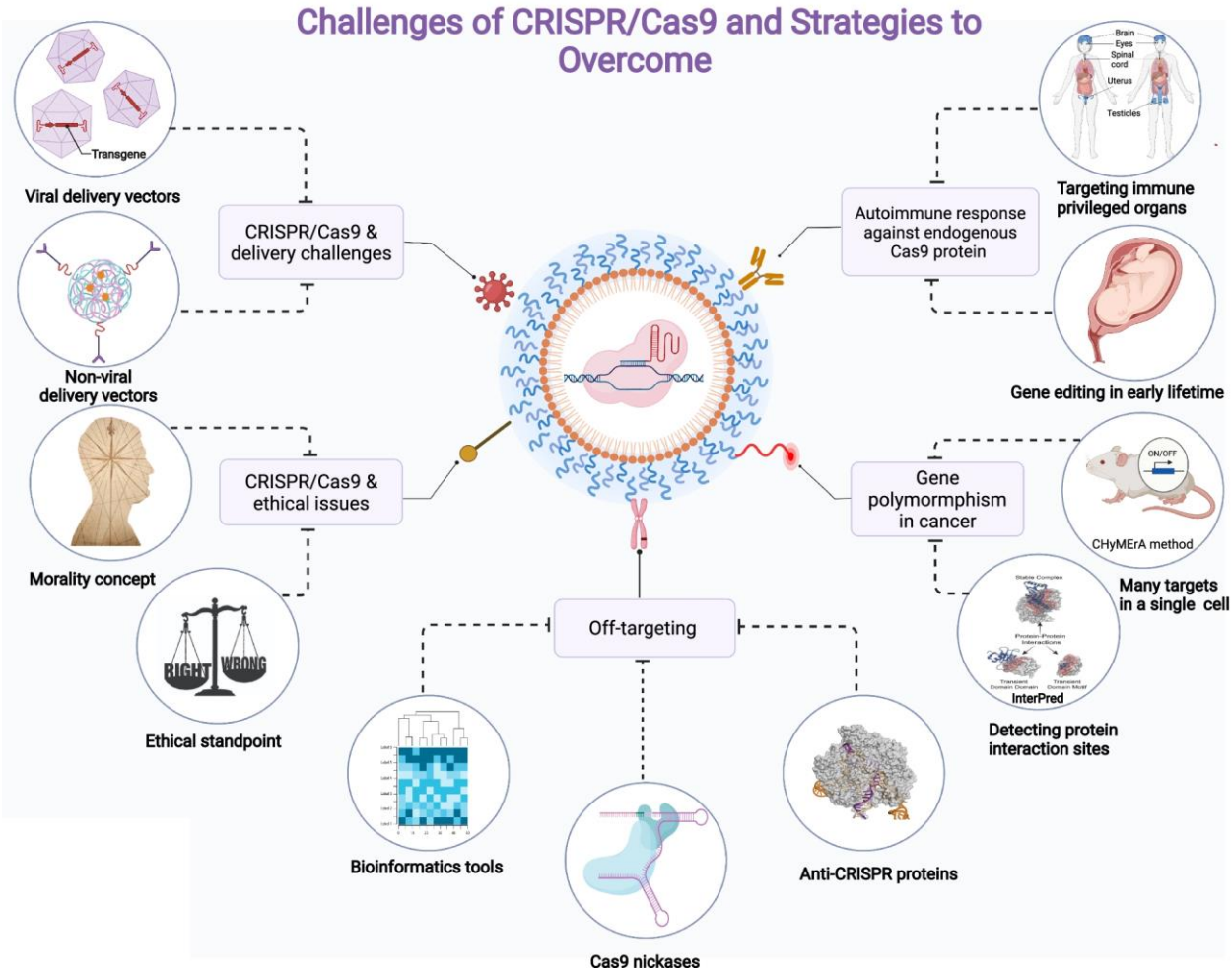

Figure 9 Challenges of CRISPR/Cas9 and strategies to overcome them, integrating delivery barriers (viral/non-viral vectors), immunogenicity (pre-existing and induced responses), off-targeting, gene polymorphism/context, multi-target designs, and ethical consider considerations; mitigation approaches include immune-privileged targeting, early-lifetime editing, high-fidelity/paired-nickase designs, anti-CRISPR proteins, and bioinformatics-guided gRNA design. Adapted from Rasul et al. (2022), CC BY 4.0

6.2. Delivery barriers and immunogenicity

Delivery remains one of the principal rate-limiting factors for nearly all therapeutic CRISPR applications. Efficient intracellular transport of the Cas nuclease and guide RNA must achieve the correct stoichiometry, cellular targeting, and temporal control, all while minimizing immunogenicity. Early transfection approaches, plasmid DNA, electroporation, or viral vectors, proved efficient in cultured cells but impractical for in-vivo systems because of limited tissue penetration and potential genomic integration (Lino et al., 2018).

Lipid nanoparticles (LNPs) have since emerged as the most clinically advanced non-viral platform. Their success in *in vivo* editing of the transthyretin (TTR) gene in human hepatocytes (Gillmore et al., 2021) demonstrated transient Cas9 expression with minimal off-target editing.

However, LNP uptake is largely confined to the liver, leaving other tissues under-served. Modifications such as cationic ionizable lipids, PEGylation, and ligand decoration are being explored to expand tropism toward pulmonary, muscular, or hematopoietic compartments (Kulkarni et al., 2021).

Viral vectors, notably adeno-associated virus (AAV) and lentivirus, still dominate in laboratory models because of high transduction efficiency, but they introduce unique biosafety issues. AAV's limited packaging capacity constrains full-length Cas9 cargo, whereas lentiviral integration poses insertional-mutagenesis risk (Ran et al., 2015). Hybrid approaches using split-Cas systems or self-complementary AAV genomes partly mitigate these challenges but complicate manufacturing.

Beyond delivery efficiency, immunogenicity represents a persistent obstacle. Many Cas proteins derive from human-associated bacteria such as *Streptococcus pyogenes* and *Staphylococcus aureus,* to which most adults possess pre-existing antibodies or T-cell responses (Charlesworth et al., 2019). Exposure can trigger inflammation or rapid clearance of CRISPR components, reducing efficacy. Strategies under investigation include using orthologs from non-pathogenic species (e.g., *Neisseria meningitidis* Cas9), transient RNA or RNP delivery to minimize exposure, and encapsulation within biomimetic membranes that cloak bacterial epitopes (Wang et al., 2023).

For diagnostic contexts, delivery barriers are replaced by sample-matrix effects: viscous or inhibitor-rich materials such as blood and sputum interfere with amplification or collateral cleavage, demanding optimized lysis and buffer chemistry (Arizti-Sanz et al., 2020). Whether therapeutic or analytical, overcoming delivery limitations remains central to CRISPR's scalability and global impact.

Table 6 Delivery platforms used for CRISPR-based therapeutics and antimicrobial applications, showing cargo format, strengths, limitations, and representative use cases.

Delivery Method	**CRISPR Cargo Type**	**Key Advantages**	**Major Limitations**	**Representative Application**

AAV (Adeno-associated virus)	Cas DNA + gRNA cassette	Long-term expression, tissue tropism options	Small cargo limit (~4.7 kb), pre-existing immunity	In vivo Cas9 editing in liver and eye disorders
Lipid nanoparticles (LNPs)	Cas mRNA + gRNA or RNP	Transient expression, clinically validated, scalable	Liver tropism bias, extrahepatic delivery still limited	NTLA-2001 (first in-human CRISPR therapy), Cas13 antivirals
Engineered bacteriophages	Cas plasmid or RNP	Natural targeting of bacterial hosts, high specificity	Narrow host range, ecological release concerns	Phage-delivered Cas9 against MRSA
Conjugative plasmids	Cas cassette (DNA)	Self-spreading inside microbial communities	Horizontal gene transfer risks, biocontainment needed	Vancomycin-resistant Enterococcus plasmid curing
Exosomes / Extracellular vesicles (EVs)	Cas RNP	Low immunogenicity, potentially crosses some barriers	Loading efficiency + scalable production are challenges	Preclinical delivery of antiviral Cas13 RNPs
Polymeric / Inorganic nanoparticles	RNP or DNA	Physicochemical tunability, stable storage	Lower transfection efficiency vs LNPs	Experimental antimicrobial and cancer CRISPR delivery
Physical delivery (electroporation, microinjection)	DNA, mRNA, or RNP	High efficiency in ex vivo or localized contexts	Not scalable in vivo, tissue damage risk	Ex vivo T-cell engineering, local therapeutic delivery

6.3. Viral and microbial resistance

Just as antibiotics select for resistant bacterial subpopulations, CRISPR-based interventions exert evolutionary pressure on target genomes. Viruses and bacteria can escape CRISPR interference through point mutations, deletions, or recombination events in the protospacer or PAM sequence, changes that prevent guide recognition (Wang et al., 2019). Laboratory studies

with bacteriophages and eukaryotic viruses show that resistance can arise within a few replication cycles if only a single guide RNA is used.

To counter this, multiplexed guide designs targeting multiple conserved loci have become the default approach (Abbott et al., 2020). When a virus would need simultaneous mutations at several essential sites to evade recognition, the probability of escape drops exponentially. Another strategy involves targeting functionally constrained regions of viral genomes, such as polymerase motifs or structural-protein coding sequences, where mutations are deleterious to viral fitness. Computational surveillance pipelines now integrate these constraints to update guide libraries dynamically as new sequence data emerge (Li et al., 2023).

Microbes present analogous issues. In bacterial populations, spontaneous mutations in protospacers or acquisition of anti-CRISPR (Acr) proteins can neutralize CRISPR activity. Acrs, encoded by bacteriophages or plasmids, bind directly to Cas nucleases and inhibit target cleavage (Bondy-Denomy et al., 2015). Continuous discovery of new Acr families underscores an evolutionary arms race between CRISPR systems and their viral adversaries. Synthetic-biology efforts are now focusing on Acr-resistant Cas variants or conditional expression systems that toggle nuclease activity to outmaneuver Acr inhibition (Watters et al., 2023).

Evolutionary escape is therefore not a flaw unique to CRISPR but an inherent feature of selective biological systems. Maintaining effectiveness will require surveillance frameworks akin to antimicrobial-resistance monitoring, tracking mutation frequencies, sharing sequence data, and updating CRISPR constructs accordingly. The intersection of molecular engineering and population-level genomics will define the durability of CRISPR-based defenses in both medicine and biodefense.

6.4. Biosafety and ethical governance

The dual-use potential of CRISPR, its capacity for both beneficial and hazardous manipulation of biological systems, places biosafety and ethical governance at the center of policy debates. In therapeutic contexts, somatic editing raises concerns about unintended germline modification, mosaicism, and off-target mutagenesis, each with implications for heritable change. Following the 2018 announcement of unauthorized germline-edited infants in China, most scientific organizations reinforced global moratoria on human germline interventions pending societal consensus and stronger oversight (Baylis, 2019).

In microbial or ecological applications, biosafety concerns shift toward containment and control. Phage-delivered or conjugative CRISPR constructs introduced into environmental or clinical settings may spread beyond intended hosts. Regulatory bodies such as the U.S. NIH Recombinant DNA Advisory Committee and the European Food Safety Authority now require containment and genetic safeguards, such as self-limiting "kill switches," sequence barcodes for traceability, and strict validation of host range (Esvelt, 2023). Internationally, the Cartagena Protocol on Biosafety provides a baseline framework, but national guidelines differ in scope, underscoring the need for harmonized global governance of gene-editing tools.

Public engagement is equally vital. Transparency regarding guide sequences, delivery methods, and validation data can mitigate misinformation and build societal trust. Ethical oversight should therefore include multidisciplinary review boards that integrate molecular biologists, clinicians, bioethicists, and public representatives. CRISPR's transformative power demands governance systems as adaptive as the technology itself.

6.5. Regulatory uncertainties

CRISPR-based therapeutics and diagnostics challenge existing regulatory paradigms because they combine properties of biological products, devices, and software. In the United States, the Food and Drug Administration (FDA) classifies genome-editing therapies under the category of gene therapy products, requiring Investigational New Drug (IND) applications with detailed data on vector design, off-target analysis, and biodistribution (U.S. Food and Drug Administration, 2023). However, the rapid pace of innovation, especially for modular, programmable designs, creates tension between regulatory caution and the need for agility during outbreaks or personalized therapy development.

In diagnostics, the situation is even more complex. While emergency-use authorizations during the COVID-19 pandemic established precedents for CRISPR-based assays, permanent classification and reimbursement pathways remain inconsistent (U.S. Food and Drug Administration, 2022). The World Health Organization and regional regulators are now exploring risk-based frameworks that balance safety with innovation, potentially introducing adaptive licensing models where assays can evolve with validated guide updates instead of full re-approval.

Developing uniform international standards will be critical. Regulatory fragmentation could impede technology transfer and global health equity, particularly if licensing costs and validation requirements differ widely between jurisdictions. Collaborative regulatory science

initiatives that share reference standards, metrology tools, and validation data may streamline approval processes without compromising safety.

6.6. Economic and infrastructural limitations

The promise of CRISPR technology is tempered by economic and infrastructural disparities that influence access and sustainability. Manufacturing high-purity Cas proteins, synthetic guide RNAs, and quality-controlled delivery materials remains cost-intensive. Although prices have declined sharply since 2015, large-scale deployment, especially in low- and middle-income countries, still faces logistical bottlenecks in reagent cold-chain maintenance, assay standardization, and technician training (WHO, 2022).

In therapeutics, costs escalate with individualized design and regulatory compliance. Each new CRISPR therapeutic may require bespoke validation of off-target risk and immune response, leading to development timelines comparable to those of small-molecule drugs (Eisenstein, 2020). Intellectual-property fragmentation adds another barrier: multiple overlapping patents on Cas enzymes, guide design algorithms, and delivery methods complicate licensing for smaller biotechnology firms. Initiatives promoting open-access toolkits and public–private consortia are essential to democratize innovation.

In diagnostics, the situation is improving faster. Lyophilized, room-temperature-stable CRISPR kits reduce distribution costs and bypass refrigeration, but scaling such production still requires regional manufacturing hubs and technology transfer agreements. Building local capacity for reagent synthesis and assay assembly will determine whether CRISPR diagnostics achieve sustainable penetration beyond research laboratories.

6.7. Summary and outlook

Each limitation discussed, specificity, delivery, resistance, governance, regulation, and economics, highlights the gap between laboratory potential and practical deployment. CRISPR's maturation will depend less on single scientific breakthroughs than on systems-level integration: predictive computational models to minimize off-targets, efficient and tissue-specific delivery architectures, globally harmonized biosafety standards, and equitable access strategies that prevent technological monopolization.

The field is already progressing toward these goals. Machine-learning-driven guide design, compact and immune-silent nucleases, and standardized validation pipelines are gradually resolving technical uncertainties. Ethical and regulatory frameworks, while uneven, are

beginning to stabilize through international dialogue. Ultimately, CRISPR's sustainability will hinge on embedding safety, transparency, and inclusivity as foundational design principles, ensuring that the same system capable of editing genes also strengthens humanity's collective defense against biological threats.

Table 7 Key scientific, translational, and regulatory challenges associated with CRISPR deployment, along with representative mitigation strategies under investigation.

Challenge	Domain(s) Affected	Root Cause	Mitigation Strategy	Development Status
Off-target editing	Therapeutics, antimicrobials	Mismatch tolerance, PAM flexibility	High-fidelity Cas variants, improved guide design, genome-wide off-target profiling	Partially resolved, active refinement
Immune response to Cas proteins	In vivo therapeutics	Pre-existing antibodies to SpCas9 / SaCas9	Cas ortholog substitution, transient mRNA/RNP delivery, immune shielding	Ongoing mitigation
Viral or bacterial escape mutations	Antivirals, antimicrobials	High mutation rate at protospacers	Multiplexed guides, conserved-site targeting, real-time sequencing surveillance	Effective in vitro, early in vivo tests
Horizontal gene transfer of CRISPR constructs	Antimicrobials (phage / plasmid systems)	Mobility of genetic elements	Self-deleting circuits, restricted host-range vectors, kill-switch designs	Early-stage synthetic biology solutions
Collateral cleavage saturation	Diagnostics (Cas12/Cas13)	Uncontrolled reporter cleavage after activation	One-pot reaction chemistry, confinement in droplets, engineered low-collateral variants	Actively optimized

Delivery constraints	All therapeutic domains	Cas gene size, payload limits, tropism	Mini-Cas systems, LNPs, EVs, split-Cas designs	Major bottleneck, improving
Regulatory uncertainty	Therapeutics, diagnostics, antimicrobials	Classification overlap (drug/biologic/device)	FDA EUA precedents, adaptive licensing, WHO guidance frameworks	Evolving
Cost & access barriers	Global deployment	Reagent cost, cold-chain dependence	Lyophilized kits, open-source tools, decentralized bio-manufacturing	In progress

Chapter 7

CRISPR in Biodefense and Biosecurity

The extraordinary precision and accessibility of CRISPR–Cas systems have transformed biotechnology from a specialized research field into a globally distributed capability. While this democratization accelerates scientific progress, it also introduces complex biosecurity considerations. The same properties that make CRISPR invaluable for pathogen detection, vaccine development, and therapeutic innovation could, if misused or inadequately regulated, facilitate the design or enhancement of biological agents (DiEuliis & Giordano, 2020). Understanding this dual-use nature, where CRISPR serves as both a shield and a sword, is essential for developing responsible governance frameworks and biodefense applications that protect rather than endanger global health.

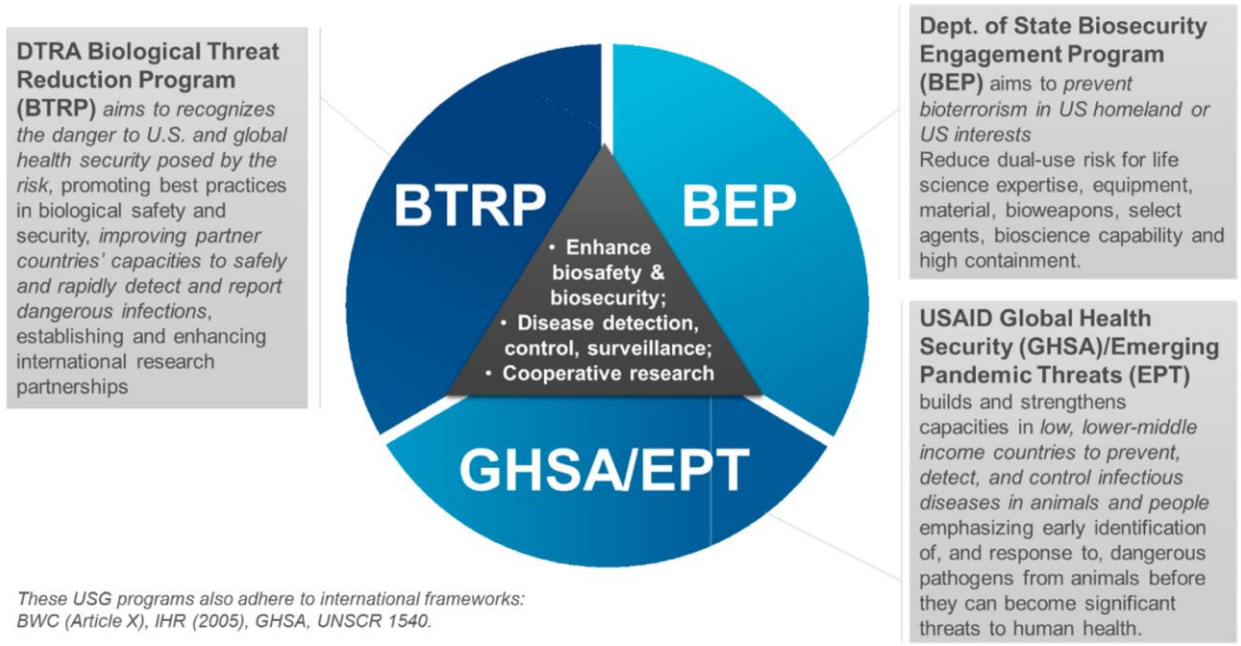

Figure 10 Biosecurity and biodefense program architecture illustrating how the U.S. DoD Biological Threat Reduction Program (BTRP), State Department Biosecurity Engagement Program (BEP), and USAID GHSA/EPT coordinate to enhance biosafety/biosecurity, disease detection and control, surveillance, and cooperative research. Adapted from Yeh et al. (2019), licensed under CC BY 4.0

7.1. Dual-use potential and emerging risks

CRISPR's capacity to modify genomes with high precision and relatively low technical barriers makes it a quintessential dual-use technology. Unlike traditional recombinant-DNA methods requiring extensive expertise and infrastructure, CRISPR allows even modestly equipped

laboratories to manipulate viral or bacterial genomes efficiently. Potential misuse scenarios include the reconstruction of extinct pathogens, the enhancement of virulence or transmissibility, or the circumvention of existing immune protections (Esvelt, 2023).

The synthesis of horsepox virus in 2017 using publicly available genomic data and mail-order DNA exemplified the feasibility of reconstructing complex viral pathogens outside high-containment facilities (Noyce et al., 2018). CRISPR could theoretically simplify such efforts by providing programmable editing tools to accelerate genome assembly, integrate virulence factors, or test resistance to antiviral countermeasures. While such scenarios remain speculative, they highlight how CRISPR reduces both the cost and the skill threshold for genetic modification, expanding the potential actor base beyond state-sponsored programs.

At the same time, the legitimate research that uses CRISPR to understand pathogen biology or improve biosurveillance could inadvertently generate sensitive information that, if released without contextual safeguards, might be repurposed. The global research community therefore faces a delicate balance between open science and responsible disclosure, a challenge reminiscent of early debates surrounding nuclear physics and cybersecurity.

7.2. CRISPR for pathogen detection and surveillance

Paradoxically, the same programmable specificity that raises dual-use concerns also underpins CRISPR's most valuable biodefense contribution, real-time pathogen detection and genomic surveillance. Cas12- and Cas13-based diagnostics such as SHERLOCK and DETECTR have demonstrated the ability to identify viral, bacterial, and toxin-producing agents rapidly, making them indispensable for outbreak response and environmental biosurveillance (Broughton et al., 2020; Arizti-Sanz et al., 2020). Their portability, low cost, and independence from centralized laboratories allow use in remote or resource-limited settings where traditional PCR infrastructure is unavailable.

In biodefense frameworks, CRISPR diagnostics can function as front-line biosentinels, capable of detecting early pathogen emergence before clinical symptoms spread widely. For example, environmental sampling combined with multiplexed CRISPR sensors could identify engineered or natural pathogens through characteristic genetic signatures. During the COVID-19 pandemic, CRISPR-based platforms were rapidly reconfigured for variant identification, demonstrating their suitability for continuous genomic monitoring (Patchsung et al., 2021). Similar workflows can be adapted for surveillance of high-consequence pathogens such as *Bacillus anthracis* or *Yersinia pestis* in environmental matrices.

Integration with digital biosurveillance networks transforms CRISPR diagnostics into data-generating nodes. Coupled with mobile sequencing and cloud analytics, these devices could support global early-warning systems that track mutations, antimicrobial-resistance determinants, and unusual genomic patterns indicative of laboratory modification (DiEuliis & Giordano, 2020). Such capabilities align with the “One Health” paradigm, linking human, animal, and environmental pathogen monitoring through shared genetic intelligence.

7.3. Countermeasure development and vaccine design

Beyond detection, CRISPR contributes to biodefense through rapid countermeasure development. Genome-editing platforms accelerate functional studies of pathogen virulence genes, host susceptibility factors, and immune-evasion mechanisms, providing essential data for rational vaccine or therapeutic design (Komor et al., 2016). In model systems, CRISPR knockout libraries have been employed to identify host dependency genes exploited by influenza, flaviviruses, and coronaviruses, guiding the development of host-targeted antivirals with reduced risk of resistance (Wei et al., 2020).

In vaccine development, CRISPR enhances both the speed and precision of antigen design. Editing viral or bacterial genomes allows for attenuation through targeted gene deletions or the insertion of immune-modulating elements, expediting the generation of live-attenuated or vector-based vaccines (Gao et al., 2020). Moreover, CRISPR enables the construction of synthetic vaccine platforms in which immune epitopes from multiple pathogens are expressed within a single chassis organism or nanoparticle scaffold, potentially revolutionizing preparedness against novel or engineered threats.

CRISPR-based screening tools have also been pivotal in identifying immune-evasion variants of concern before their widespread emergence. Genome-wide CRISPR interference (CRISPRi) and activation (CRISPRa) libraries in immune cell lines are increasingly used to study viral entry, replication, and cytokine responses, thereby informing immunomodulatory drug design (Schmidt et al., 2021). These approaches shorten the timeline between pathogen discovery and countermeasure validation, a capability critical in biodefense scenarios where speed dictates containment success.

7.4. Governance and international regulation

Effective biodefense depends not only on technology but also on governance. The dual-use nature of CRISPR has intensified discussions within international arms-control frameworks such as the Biological Weapons Convention (BWC), which prohibits development or stockpiling of biological weapons. Although the BWC lacks a formal verification regime, recent meetings of state parties have acknowledged the need to address synthetic biology and genome-editing technologies explicitly (United Nations Office for Disarmament Affairs [UNODA], 2022).

National security agencies have begun integrating CRISPR into biosecurity risk-assessment models, evaluating how access to genome-editing kits, DNA synthesis services, and cloud-based design tools could be misused. Countries including the United States, United Kingdom, and members of the European Union now require DNA synthesis providers to screen orders for sequences associated with controlled pathogens or toxins (DiEuliis & Giordano, 2020). Parallel efforts promote "gene-drive governance," establishing oversight for CRISPR systems capable of altering wild populations, ensuring that ecological interventions are confined, reversible, and transparent to affected communities (Esvelt, 2023).

Global cooperation remains uneven. Many low- and middle-income nations lack formal oversight mechanisms for advanced biotechnologies despite increasing access to CRISPR reagents through international collaborations. Establishing global standards for biosafety training, information sharing, and incident reporting is therefore urgent. The World Health Organization's Global Guidance Framework for Responsible Life Sciences (WHO, 2022) provides a starting template, emphasizing risk-benefit analysis, transparency, and education.

7.5. CRISPR-based biosecurity monitoring and forensic applications

CRISPR technologies are also being explored as tools for bioforensics and incident attribution, offering precision in identifying the genetic origin or modification history of biological agents. Because CRISPR editing leaves unique molecular signatures, such as PAM scars, noncanonical repair junctions, or guide RNA target residues, these can serve as traceable markers distinguishing natural evolution from synthetic manipulation (DiEuliis & Giordano, 2020). Establishing standardized pipelines for forensic genomic analysis could therefore enhance transparency and accountability under the Biological Weapons Convention and related treaties.

In parallel, environmental biosurveillance systems equipped with multiplexed CRISPR sensors can continuously monitor air, water, or surface samples for pathogen nucleic acids. When integrated with microfluidic concentrators and wireless data transmission, these sensors could function as distributed "genetic smoke alarms," alerting authorities to potential accidental or deliberate releases. Several defense research agencies have funded early prototypes of such systems for field deployment, capable of detecting aerosolized anthrax simulants or influenza RNA within minutes (Esvelt, 2023).

CRISPR's programmability further enables *adaptive surveillance*: new guides can be uploaded remotely to update detector arrays in response to emerging threats. This capacity mirrors cybersecurity principles, regular patching against new vulnerabilities, positioning CRISPR biosensors as an analogue to real-time antivirus software for the biological domain. However, these same capabilities raise data security and privacy concerns, as genomic information from environmental monitoring must be protected from misuse or overreach. International standards for data anonymization and ethical data sharing will therefore be crucial to prevent surveillance misuse under the guise of health protection.

7.6. Ethical, societal, and strategic considerations

The intersection of CRISPR and biodefense introduces profound ethical questions. On one hand, it empowers public health through early detection and rapid countermeasure development; on the other, it risks normalizing dual-use research of concern. Policymakers must ensure that biodefense investments do not inadvertently stimulate arms-race dynamics in biotechnology (DiEuliis & Giordano, 2020). Transparency, international collaboration, and civilian oversight should remain cornerstones of CRISPR-related biodefense programs.

Education plays a critical preventive role. Embedding biosecurity literacy into molecular biology curricula can sensitize scientists to dual-use dilemmas and responsible research conduct. Community laboratories and do-it-yourself biology spaces, where CRISPR tools are increasingly accessible, require particular attention. Codes of conduct, biosafety audits, and voluntary registration systems can preserve openness in citizen science while mitigating misuse.

Equity and trust are equally important. If CRISPR-based biodefense tools are developed solely within military or high-income-country frameworks, they may reinforce global asymmetries in biosafety capacity. Collaborative frameworks that share protocols, reference materials, and data openly with low-resource regions can ensure collective rather than exclusive security. The

concept of “biosecurity as a global public good” underscores that no country can achieve true biological safety in isolation (WHO, 2022).

7.7. Summary and outlook

CRISPR’s role in biodefense embodies the dual imperative of innovation and restraint. As a shield, it enhances global readiness through rapid diagnostics, surveillance, and the design of countermeasures. As a sword, it poses risks if exploited for offensive or uncontrolled applications. The challenge lies in maintaining equilibrium between these two facets through governance frameworks that evolve as rapidly as the technology itself.

Future progress will depend on establishing transparent international norms that distinguish defensive preparedness from offensive capability. Integrating CRISPR-based detection and sequencing with artificial intelligence, cloud networks, and portable hardware can create real-time biosurveillance architectures that deter misuse by enhancing early detection. Parallel development of ethical guidelines, verification mechanisms, and secure data infrastructures will anchor these systems in trust and accountability.

If managed responsibly, CRISPR could become the foundation of a new era of **proactive biodefense**, one focused on prevention, resilience, and global cooperation rather than deterrence through secrecy. In this vision, the same technology that once raised fears of bioterrorism becomes an instrument of transparency, enabling societies to see, respond to, and contain biological threats before they escalate.

Chapter 8

Future Directions and Emerging Frontiers

The rapid evolution of CRISPR technology continues to reshape the molecular life sciences, transcending its original role as a bacterial immune mechanism to become a universal platform for genome modulation, pathogen defense, and biosensing. The next phase of CRISPR innovation will likely be defined not by incremental improvement, but by convergence, with artificial intelligence, synthetic biology, nanotechnology, and global public health infrastructures. Together, these intersections will determine how CRISPR moves from experimental promise to a mature pillar of 21st-century medicine and biodefense (Koonin & Makarova, 2022; Esvelt, 2023).

8.1. Novel effectors and next-generation Cas systems

The ongoing discovery of new CRISPR–Cas systems expands the functional toolkit available to researchers. Cas9 and Cas12 dominated early applications, but recent metagenomic exploration has uncovered compact and orthogonal effectors such as CasΦ (Cas12j), CasMINI, and CasΛ, which are small enough to fit within standard AAV vectors while retaining programmable activity (Pausch et al., 2020; Xu et al., 2021). These miniature nucleases offer significant advantages for therapeutic delivery, particularly for in vivo editing, where packaging constraints limit the use of large constructs.

Parallel work on RNA-targeting systems, including Cas13 variants and the recently characterized Cas7-11, promises improved control over transcript-level regulation with minimal collateral cleavage (Ozcan et al., 2021). Beyond editing, base and prime editors that fuse Cas proteins with deaminases or reverse transcriptases now enable single-base or templated changes without double-strand breaks (Anzalone et al., 2019). The development of “compact prime editors” and “RNA-guided transposases” further suggests a future where precise genetic writing and insertion occur without the risks of classical cleavage-based repair.

Engineered Cas effectors are also being optimized for temperature tolerance, immune evasion, and multiplex programmability, extending their usability across diverse species and cell types. Collectively, these innovations move CRISPR closer to a universal molecular Swiss Army knife, capable of editing, sensing, and regulating with context-specific precision.

8.2. AI-driven CRISPR design and automation

As the scale of CRISPR applications expands, artificial intelligence (AI) and machine learning are becoming indispensable for guide design, off-target prediction, and data interpretation. Conventional design algorithms rely on sequence heuristics, but deep-learning models trained on large editing datasets now outperform them by capturing complex nucleotide-context dependencies and chromatin effects (Kim et al., 2023).

Modern platforms such as DeepCRISPR, CRISPR-Net, and Azimuth 2.0 integrate sequence, epigenetic, and structural features to forecast both efficiency and specificity simultaneously (Xue et al., 2021). These models enable automated selection of optimal guide RNAs within minutes, a process that once required laborious empirical screening. In diagnostics, AI assists in identifying conserved genomic motifs suitable for multiplex detection and in optimizing crRNA–reporter pairings for maximal signal-to-noise ratios (Li et al., 2023).

Automation extends beyond design to experimental execution. Robotic liquid-handling systems and microfluidic arrays can perform thousands of CRISPR experiments in parallel, generating standardized data to refine AI models in a self-reinforcing loop. This “closed-loop design-build-test-learn” framework, central to synthetic-biology pipelines, promises near-real-time optimization of CRISPR constructs for therapeutic, antimicrobial, and diagnostic applications. When combined with cloud-based repositories, it lays the groundwork for global CRISPR design networks that continuously update and validate new guide libraries for emerging pathogens.

8.3. Integrated delivery systems and synthetic-biology convergence

The convergence of CRISPR with nanotechnology and synthetic-biology chassis design is producing hybrid delivery systems that blur the lines between biological and mechanical vectors. Next-generation lipid nanoparticles incorporate modular components such as pH-responsive lipids and cell-targeting peptides, allowing spatiotemporal control of CRISPR activity (Kulkarni et al., 2021). At the same time, engineered viral-like particles and exosome-mimetic vesicles offer biocompatible platforms for delivering Cas proteins and RNA guides without genomic integration (Lino et al., 2018).

Synthetic biology contributes an additional layer of precision through programmable gene circuits. Cas effectors can be embedded within regulatory networks that activate only in specific cellular environments, such as hypoxia, inflammation, or pathogen-specific signals,

thereby reducing off-target exposure and systemic toxicity (Liu et al., 2023). These “smart CRISPR therapeutics” exemplify the shift toward *context-aware editing*, in which editing activity is dynamically modulated by the cellular state.

The same logic applies to diagnostics. Synthetic circuits combining CRISPR sensors with toehold switches or aptamer-based modules create highly specific biosensors capable of logical operations (“AND”/“OR” gates) for multi-analyte detection (Tian et al., 2022). Integrating such programmable biosensors into portable lab-on-chip devices will enable continuous health monitoring, environmental surveillance, and biosecurity applications, all operating autonomously under predefined safety constraints.

8.4. Clinical translation and regulatory evolution

The maturation of CRISPR therapeutics will hinge on regulatory models that accommodate programmable and modular interventions rather than static chemical entities. Traditional drug-approval pathways assume a fixed molecular composition, yet CRISPR constructs can be reprogrammed with new guide RNAs to treat different diseases or viral variants. Regulators are therefore moving toward platform-based oversight, in which a core editing system (e.g., Cas enzyme and delivery vehicle) is preapproved, and subsequent guide updates undergo accelerated review (U.S. Food and Drug Administration, 2023).

Early human trials have already validated in vivo gene editing safety. The NTLA-2001 study demonstrated durable TTR knockdown following a single infusion of Cas9 mRNA and guide RNA using lipid nanoparticles (Gillmore et al., 2021). Similar frameworks are being extended to ex vivo therapies for hemoglobinopathies and immunodeficiencies. The next frontier will involve episomal or transient editing, using RNA or RNP formulations that minimize long-term genomic exposure while achieving therapeutic efficacy. These transient approaches are particularly promising for antiviral or antimicrobial applications, where reversible immunity is desirable.

Parallel progress is occurring in diagnostics. Following their emergency-use authorizations, CRISPR diagnostic assays are moving toward full regulatory clearance and international standardization. Agencies including the FDA, WHO, and European Medicines Agency are exploring harmonized performance benchmarks, defining sensitivity, reproducibility, and biosafety criteria specific to CRISPR-based assays (WHO, 2023). Such frameworks will accelerate global adoption by establishing clear, predictable validation routes.

8.5. Global equity, open science, and sustainable access

For CRISPR technologies to fulfill their humanitarian potential, they must be distributed equitably. The decreasing cost of reagents and proliferation of open-access design tools are narrowing historical gaps between high- and low-income research systems. Initiatives such as **OpenCRISPR**, the **Addgene Cas Repository**, and WHO's **Technology Access Pool (C-TAP)** provide shared protocols, plasmids, and datasets for institutions lacking commercial licensing power (WHO, 2022).

Nevertheless, systemic disparities persist. The majority of CRISPR clinical trials are conducted in North America, Europe, and China, while many regions facing endemic infectious diseases have minimal capacity for CRISPR research or validation. Addressing this requires not only reagent donation but also **infrastructure transfer**, regional reagent manufacturing, cold-chain independence through lyophilized formulations, and training programs in bioinformatics, biosafety, and regulatory compliance (Esvelt, 2023).

Sustainable access also depends on intellectual-property reform. The fragmentation of Cas enzyme patents across multiple universities and corporations complicates licensing for low-resource contexts. Proposals for **patent pools** or **nonexclusive humanitarian licenses** could balance innovation incentives with public-good access. Furthermore, embedding CRISPR into open-science ecosystems will strengthen transparency, reproducibility, and trust, critical elements for global collaboration in areas such as pathogen surveillance and biodefense.

8.6. Synthesis and future outlook

The convergence of CRISPR innovation across molecular engineering, computation, and governance is shaping a new paradigm of **adaptive biotechnologies**, systems that sense, learn, and respond to biological change with unprecedented speed. Within the next decade, we are likely to witness the emergence of autonomous CRISPR platforms capable of simultaneous diagnosis, intervention, and monitoring, supported by AI-guided design and globally networked data infrastructures.

However, technological capability must advance in tandem with ethical and policy frameworks. Transparent regulation, equitable access, and international oversight will determine whether CRISPR evolves as a cooperative shield for humanity or fragments into proprietary, asymmetrical power structures. Integrating these principles early, safety, openness,

and solidarity, will ensure that CRISPR's trajectory strengthens public health and biosafety rather than undermining them.

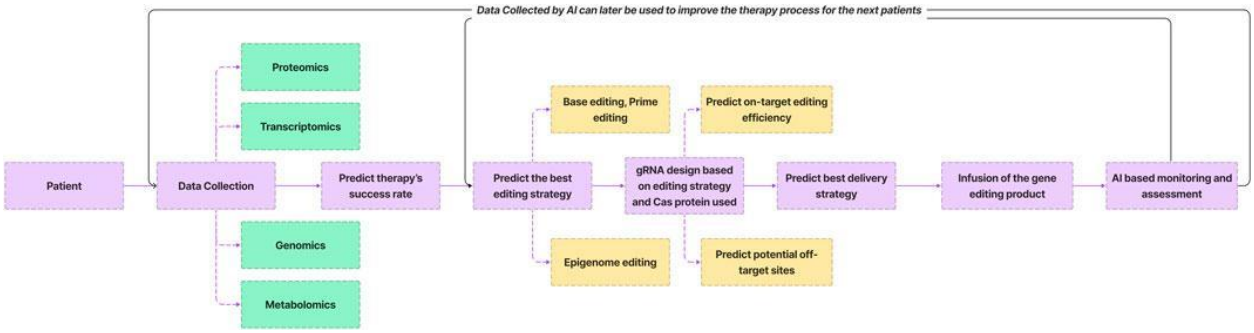

Figure 11 Convergence roadmap toward adaptive CRISPR platforms: multi-omics data collection → AI-guided gRNA and editor selection (base/prime/epi) → delivery strategy optimization → infusion and real-time AI monitoring, with feedback improving subsequent iterations. Adapted from Dixit et al. (2024), CC BY 4.0.

Chapter 9

Conclusion

Over the past decade, CRISPR–Cas systems have transformed the biological sciences from descriptive disciplines into programmable engineering fields. What began as a bacterial defense mechanism has become a universal toolkit for editing, sensing, and defending across all domains of life. This review traced CRISPR's evolution from its mechanistic foundations to its deployment as an antiviral, antimicrobial, diagnostic, and biodefense instrument, each application revealing both immense promise and profound responsibility.

CRISPR's power lies in its programmability and adaptability. Whether disabling viral genomes, reversing antibiotic resistance, or detecting emerging pathogens, its modularity allows near-instant reconfiguration in response to evolving biological challenges. This capacity positions CRISPR as a cornerstone of precision medicine and synthetic biology, where interventions can be designed, tested, and iterated in silico before implementation. Yet, the same accessibility that fuels innovation demands vigilant oversight to prevent misuse or unintended ecological and societal consequences.

The central challenge is therefore not only technical but philosophical: how to govern programmable biology. Achieving equitable access, transparent validation, and global safety standards will require sustained cooperation among scientists, regulators, and the public. Ethical governance must advance in parallel with molecular engineering, embedding biosafety and biosecurity into the design phase rather than treating them as downstream concerns. International frameworks, such as the WHO's Global Guidance for Responsible Life Sciences and updates to the Biological Weapons Convention, represent early steps toward this integration but remain works in progress.

Looking forward, the trajectory of CRISPR technologies points toward convergence and integration. AI-guided guide design, nanoscale delivery, and multiplexed biosensing will merge into cohesive, adaptive platforms capable of both therapeutic correction and real-time surveillance. In this emerging ecosystem, CRISPR will serve as both microscope and scalpel, illuminating genetic mechanisms while reshaping them with surgical precision. The long-term vision extends beyond treating disease to constructing resilient biological infrastructures that safeguard populations, environments, and economies from microbial threats.

In sum, CRISPR embodies the paradox of modern biotechnology: a system born from microbial immunity now entrusted with protecting humanity itself. Its success will not be measured solely by technical milestones but by how wisely, inclusively, and safely it is applied. Harnessed with foresight, CRISPR can transform the 21st century into an era where biology becomes not a source of vulnerability, but a foundation for global security and health.

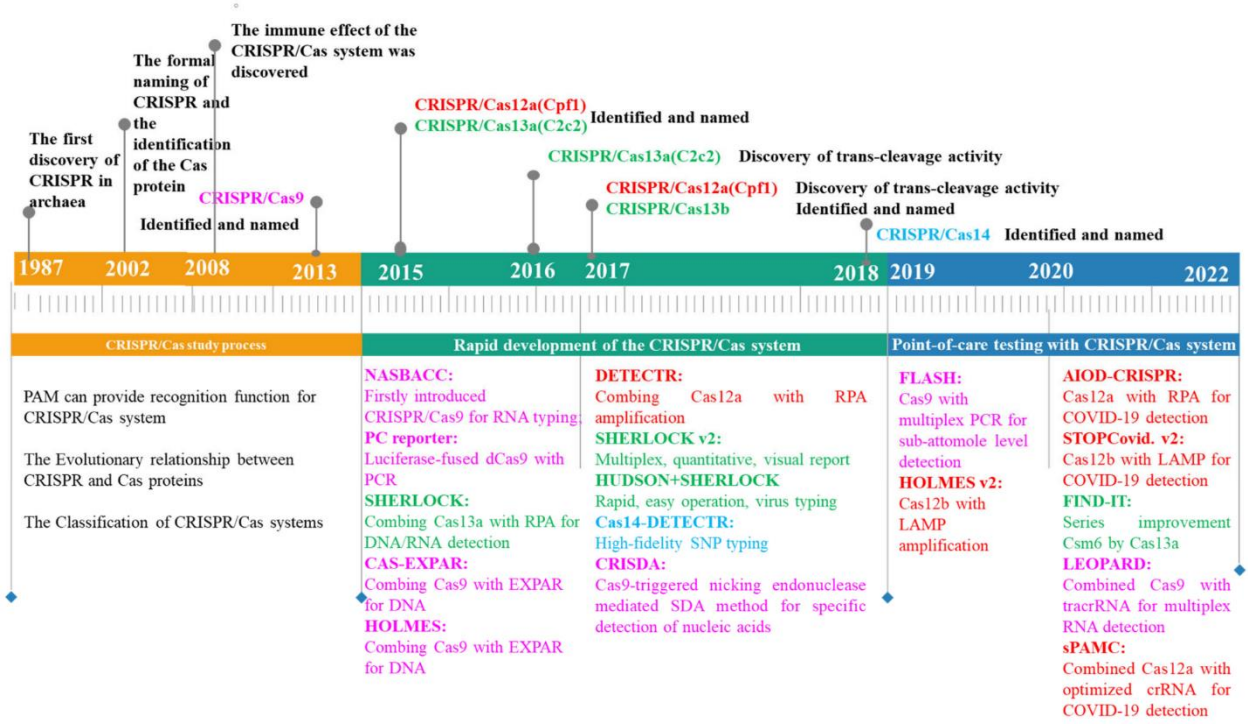

Figure 12 Timeline of CRISPR/Cas development from early discoveries (1987–2007) through rapid tool expansion (Cas9, Cas12, Cas13, Cas14) and point-of-care diagnostics (e.g., SHERLOCK, DETECTR), aligning technology milestones with translational applications. Adapted from He et al. (2023), licensed under CC BY 4.0

Reference List

1. Abbott, T. R., Dhamdhere, G., Liu, Y., Lin, X., Goudy, L., Zeng, L., … Collins, J. J. (2020). Development of CRISPR as an antiviral strategy to combat SARS-CoV-2 and influenza. *Cell*, 181(4), 865–876.e12. https://doi.org/10.1016/j.cell.2020.04.020
2. Ackerman, C. M., Myhrvold, C., Thakku, S. G., Freije, C. A., Metsky, H. C., Yang, D. K., … Sabeti, P. C. (2020). Massively multiplexed nucleic acid detection using Cas13. *Nature*, 582(7811), 277–282. https://doi.org/10.1038/s41586-020-2279-8
3. Anzalone, A. V., Randolph, P. B., Davis, J. R., Sousa, A. A., Koblan, L. W., Levy, J. M., … Liu, D. R. (2019). Search-and-replace genome editing without double-strand breaks or donor DNA. *Nature*, 576(7785), 149–157. https://doi.org/10.1038/s41586-019-1711-4
4. Arizti-Sanz, J., Freije, C. A., Stanton, A. C., Petros, B. A., Boehm, C. K., Siddiqui, S., … Sabeti, P. C. (2020). Streamlined inactivation, amplification, and Cas13-based detection of SARS-CoV-2. *Nature Communications*, 11(1), 5921. https://doi.org/10.1038/s41467-020-19097-x
5. Abudayyeh, O. O., Gootenberg, J. S., Konermann, S., et al. (2016). C2c2 is a single-component programmable RNA-guided RNA-targeting CRISPR effector. *Science*, 353(6299), aaf5573. https://doi.org/10.1126/science.aaf5573
6. Ackerman, C. M., Myhrvold, C., Thakku, S. G., et al. (2020). Massively multiplexed nucleic acid detection with Cas13 (CARMEN). *Nature*, 582, 277–282. https://doi.org/10.1038/s41586-020-2279-8
7. Arizti-Sanz, J., Freije, C. A., Stanton, A. C., et al. (2020). Streamlined, inactivation-free Cas13-based detection of SARS-CoV-2. *Nature Communications*, 11, 5921. https://doi.org/10.1038/s41467-020-19097-x
8. Azeez, S. S., Kolawole, O. O., & Adeola, O. A. (2024). Advances in CRISPR-Cas technology and its applications: Revolutionising precision medicine. *Frontiers in Genome Editing, 6,* 1509924. https://doi.org/10.3389/fgeed.2024.1509924
9. Baddeley, H. J. E., & Isalan, M. (2021). The application of CRISPR/Cas systems for antiviral therapy. *Frontiers in Genome Editing, 3*, 745559. https://doi.org/10.3389/fgeed.2021.745559
10. Baylis, F. (2019). Human germline genome editing: Do the ends justify the means? *Hastings Center Report*, 49(S1), S9–S18. https://doi.org/10.1002/hast.982
11. Bhardwaj, P., Kant, R., Behera, S. P., Dwivedi, G. R., & Singh, R. (2022). Next-generation diagnostic with CRISPR/Cas: Beyond nucleic acid detection. *International Journal of Molecular Sciences, 23*(11), 6052. https://doi.org/10.3390/ijms23116052
12. Bikard, D., Euler, C. W., Jiang, W., Nussenzweig, P. M., Goldberg, G. W., Duportet, X., … Marraffini, L. A. (2014). Exploiting CRISPR–Cas nucleases to produce sequence-specific antimicrobials. *Nature Biotechnology*, 32(11), 1146–1150. https://doi.org/10.1038/nbt.3043
13. Bondy-Denomy, J., Pawluk, A., Maxwell, K. L., & Davidson, A. R. (2015). Bacteriophage genes that inactivate the CRISPR/Cas bacterial immune system. *Nature*, 493(7432), 429–432. https://doi.org/10.1038/nature11723

14. Broughton, J. P., Deng, X., Yu, G., Fasching, C. L., Servellita, V., Singh, J., … Chiu, C. Y. (2020). CRISPR–Cas12-based detection of SARS-CoV-2. Nature Biotechnology, 38(7), 870–874. https://doi.org/10.1038/s41587-020-0513-4
15. Bikard, D., Euler, C. W., Jiang, W., et al. (2014). Exploiting CRISPR-Cas nucleases to produce sequence-specific antimicrobials. Nature Biotechnology, 32(11), 1146–1150. https://doi.org/10.1038/nbt.3043
16. Broughton, J. P., Deng, X., Yu, G., et al. (2020). CRISPR–Cas12-based detection of SARS-CoV-2 (DETECTR). Nature Biotechnology, 38(7), 870–874. https://doi.org/10.1038/s41587-020-0513-4
17. Charlesworth, C. T., Deshpande, P. S., Dever, D. P., Camarena, J., Lemgart, V. T., Cromer, M. K., … Porteus, M. H. (2019). Identification of pre-existing adaptive immunity to Cas9 proteins in humans. *Nature Medicine*, 25(2), 249–254. https://doi.org/10.1038/s41591-018-0326-y
18. Chen, J. S., Ma, E., Harrington, L. B., Da Costa, M., Tian, X., Palefsky, J. M., & Doudna, J. A. (2018). CRISPR–Cas12a target binding unleashes indiscriminate single-stranded DNase activity. *Science*, 360(6387), 436–439. https://doi.org/10.1126/science.aar6245
19. Chen, J. S., Dagdas, Y. S., Kleinstiver, B. P., Welch, M. M., Sousa, A. A., Harrington, L. B., … Doudna, J. A. (2017). Enhanced proofreading governs CRISPR–Cas9 targeting accuracy. *Nature*, 550(7676), 407–410. https://doi.org/10.1038/nature24268
20. Chertow, D. S. (2018). Next-generation diagnostics with CRISPR. *Science*, 360(6387), 381–382. https://doi.org/10.1126/science.aat4982
21. Citorik, R. J., Mimee, M., & Lu, T. K. (2014). Sequence-specific antimicrobials using efficiently delivered RNA-guided nucleases. *Nature Biotechnology*, 32(11), 1141–1145. https://doi.org/10.1038/nbt.3011
22. DiEuliis, D., & Giordano, J. (2020). Why gene editors like CRISPR could spur a new form of biological warfare, and why they shouldn't. *Health Security*, 18(6), 437–444. https://doi.org/10.1089/hs.2020.0069
23. Dixit, S., Kumar, A., Srinivasan, K., Vincent, P. M. D. R., & Ramu Krishnan, N. (2024). Advancing genome editing with artificial intelligence: Opportunities, challenges, and future directions. *Frontiers in Bioengineering and Biotechnology, 11*, 1335901. https://doi.org/10.3389/fbioe.2023.1335901
24. Duan, C., Cao, H., Zhang, L.-H., & Xu, Z. (2021). *Frontiers in Microbiology, 12*, 716064. https://doi.org/10.3389/fmicb.2021.716064
25. Eisenstein, M. (2020). Companies race to develop CRISPR therapies. *Nature Biotechnology*, 38(6), 635–638. https://doi.org/10.1038/s41587-020-0565-5
26. Esvelt, K. M. (2023). Responsible innovation in gene editing and gene drive research. *Nature Biotechnology*, 41(2), 180–189. https://doi.org/10.1038/s41587-022-01592-6
27. Fozouni, P., Son, S., de León Derby, M. D., et al. (2021). Amplification-free detection of SARS-CoV-2 with CRISPR–Cas13a and mobile phone microscopy. *Cell*, 184(2), 323–333.e9. https://doi.org/10.1016/j.cell.2020.12.001
28. Fozouni, P., Son, S., Díaz de León Derteano, C., Knott, G. J., Gray, C. N., D'Ambrosio, M. V., … Fletcher, D. A. (2021). Amplification-free detection of SARS-CoV-2 with CRISPR–Cas13a and mobile phone microscopy. *Cell*, 184(2), 323–333.e9. https://doi.org/10.1016/j.cell.2020.12.001

29. Gao, C., Wang, S., Zang, Y., Wang, C., Ji, C., & Gao, C. (2020). CRISPR/Cas9-mediated editing for viral vaccine development. *Vaccines*, 8(3), 578. https://doi.org/10.3390/vaccines8030578
30. Gillmore, J. D., Gane, E., Taubel, J., Kao, J., Fontana, M., Maitland, M. L., … Lebwohl, D. (2021). CRISPR–Cas9 in vivo gene editing for transthyretin amyloidosis. *New England Journal of Medicine*, 385(6), 493–502. https://doi.org/10.1056/NEJMoa2107454
31. Gootenberg, J. S., Abudayyeh, O. O., Lee, J. W., Essletzbichler, P., Dy, A. J., Joung, J., … Zhang, F. (2017). Nucleic acid detection with CRISPR–Cas13a/C2c2. *Science*, 356(6336), 438–442. https://doi.org/10.1126/science.aam9321
32. Gillmore, J. D., Gane, E., Taubel, J., et al. (2021). CRISPR-Cas9 in vivo gene editing for transthyretin amyloidosis (NTLA-2001). *New England Journal of Medicine*, 385, 493–502. https://doi.org/10.1056/NEJMoa2107454
33. Gootenberg, J. S., Abudayyeh, O. O., Lee, J. W., et al. (2017). Nucleic acid detection with CRISPR–Cas13a (SHERLOCK). *Science*, 356(6336), 438–442. https://doi.org/10.1126/science.aam9321
34. Harrington, L. B., Burstein, D., Chen, J. S., et al. (2018). Programmed DNA destruction by miniature CRISPR–Cas14 enzymes. *Science*, 362(6416), 839–842. https://doi.org/10.1126/science.aav4294
35. Hajian, R., Balderston, S., Tran, T., deBoer, T., Etienne, J., Sandhu, M., … Aran, K. (2019). Detection of unamplified target genes via CRISPR–Cas9 immobilized graphene biosensor. *Nature Biomedical Engineering*, 3(6), 427–437. https://doi.org/10.1038/s41551-019-0371-x
36. He, Y., Yan, W., Long, L., Dong, L., Ma, Y., Li, C., Xie, Y., Liu, N., Xing, Z., Xia, W., & Li, F. (2023). The CRISPR/Cas system: A customizable toolbox for molecular detection. *Genes*, *14*(4), 850. https://doi.org/10.3390/genes14040850
37. Joung, J., Ladha, A., Saito, M., et al. (2020). Point-of-care testing for COVID-19 using SHERLOCK/STOPCovid. *Science Translational Medicine*, 12(556), eabc7075. https://doi.org/10.1126/scitranslmed.abc7075
38. Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A., & Charpentier, E. (2012). A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science*, 337(6096), 816–821. https://doi.org/10.1126/science.1225829
39. Joung, J., Ladha, A., Saito, M., Kim, N.-G., Woolley, A. E., Segel, M., … Zhang, F. (2020). Detection of SARS-CoV-2 with SHERLOCK one-pot testing. *New England Journal of Medicine*, 383(15), 1492–1494. https://doi.org/10.1056/NEJMc2026172
40. Kaminski, R., Chen, Y., Fischer, T., Tedaldi, E., Napoli, A., Zhang, Y., … Khalili, K. (2016). Elimination of HIV-1 genomes from human T-lymphoid cells by CRISPR/Cas9 gene editing. *Scientific Reports*, 6, 22555. https://doi.org/10.1038/srep22555
41. Kennedy, E. M., Bassit, L. C., Mueller, H., Kornepati, A. V. R., Bogerd, H. P., & Cullen, B. R. (2014). Suppression of hepatitis B virus DNA accumulation in chronically infected cells using Cas9. *Journal of Virology*, 88(19), 11997–12003. https://doi.org/10.1128/JVI.01317-14
42. Kim, D., Kim, S., Kim, S., Park, J., & Kim, J. S. (2015). Genome-wide target specificity of CRISPR–Cas9 nucleases revealed by Digenome-seq. *Nature Methods*, 12(3), 237–243. https://doi.org/10.1038/nmeth.3284

43. Kim, H., Lee, W., Lee, J., & Park, S. (2023). Deep learning-based prediction and optimization of CRISPR–Cas9 activity. *Nature Communications*, 14(1), 5119. https://doi.org/10.1038/s41467-023-40612-8
44. Koonin, E. V., & Makarova, K. S. (2022). Evolutionary plasticity and functional diversity of CRISPR–Cas systems. *Nature Reviews Microbiology*, 20(3), 141–156. https://doi.org/10.1038/s41579-021-00676-x
45. Kulkarni, J. A., Witzigmann, D., Chen, S., Cullis, P. R., & van der Meel, R. (2021). Lipid nanoparticle technology for delivery of RNA therapeutics. *Nature Reviews Drug Discovery*, 20(5), 316–344. https://doi.org/10.1038/s41573-020-00159-y
46. Lino, C. A., Harper, J. C., Carney, J. P., & Timlin, J. A. (2018). Delivering CRISPR: A review of the challenges and approaches. *Nature Methods*, 15, 467–477. https://doi.org/10.1038/s41592-018-0011-5
47. Li, H., Wang, S., & Dong, X. (2022). Emerging CRISPR–Cas genome editing and diagnostic tools for biomedicine and agriculture. *Li et al., 2022*, 1–22. https://doi.org/10.3390/genes13020007
48. Li, B., Zhao, W., Luo, X., Zhang, X., Li, R., Yang, J., & Chen, S. (2022). Multiplexed detection of pathogens using CRISPR-Cas systems: From mechanism to application. *Biosensors and Bioelectronics*, 198, 113857. https://doi.org/10.1016/j.bios.2021.113857
49. Li, H., Li, Y., Liu, H., & Zhao, J. (2023). CRISPR multiplex strategies for antiviral therapy and detection. *Frontiers in Cellular and Infection Microbiology*, 13, 1121346. https://doi.org/10.3389/fcimb.2023.1121346
50. Lino, C. A., Harper, J. C., Carney, J. P., & Timlin, J. A. (2018). Delivering CRISPR: A review of the challenges and approaches. *Drug Delivery*, 25(1), 1234–1257. https://doi.org/10.1080/10717544.2018.1474964
51. Liu, X., Tan, Y., Wang, Y., Chen, S., & Liu, D. R. (2023). Synthetic circuits for programmable CRISPR control. *Nature Chemical Biology*, 19(7), 799–809. https://doi.org/10.1038/s41589-023-01243-5
52. Nidhi, S., Tripathi, V., Oleksak, P., Kunnumakkara, A. B., et al. (2021). *Novel CRISPR–Cas systems: An updated review of the current achievements, applications, and future research perspectives. International Journal of Molecular Sciences, 22*(7), 3327. https://doi.org/10.3390/ijms22073327
53. Noyce, R. S., Lederman, S., & Evans, D. H. (2018). Construction of an infectious horsepox virus vaccine from chemically synthesized DNA fragments. *PLOS ONE*, 13(1), e0188453. https://doi.org/10.1371/journal.pone.0188453
54. Ophinni, Y., Miki, S., Hayashi, Y., & Kameoka, M. (2020). Multiplexed *tat*-targeting CRISPR-Cas9 protects T cells from acute HIV-1 infection with inhibition of viral escape. *Viruses*, *12*(11), 1223. https://doi.org/10.3390/v12111223
55. O'Neill, O. (2020). Ethical aspects of genome editing. *Philosophical Transactions of the Royal Society B*, 375(1798), 20190676. https://doi.org/10.1098/rstb.2019.0676
56. Ozcan, A., Krajeski, R. N., Ioannidi, E., Lee, B., Gardner, Z., Makarova, K. S., … Zhang, F. (2021). Programmable RNA targeting with Cas7-11. *Nature*, 597(7878), 720–725. https://doi.org/10.1038/s41586-021-03839-2
57. Patchsung, M., Jantarug, K., Pattama, A., Aphicho, K., Suraritdechachai, S., Meesawat, P., … Chuthapisith, S. (2021). Clinical validation of a Cas13-based assay for the detection of

SARS-CoV-2 RNA. *Nature Biomedical Engineering*, 5(3), 279–288. https://doi.org/10.1038/s41551-020-00603-x

58. Pausch, P., Al-Shayeb, B., Bisom-Rapp, E., Tsuchida, C. A., Li, Z., Cress, B. F., … Doudna, J. A. (2020). CRISPR–CasΦ from huge phages is a hypercompact genome editor. *Science*, 369(6501), 333–337. https://doi.org/10.1126/science.abb1400
59. Peters, J. M., Colavin, A., Shi, H., Czarny, T. L., Larson, M. H., Wong, S., … Gross, C. A. (2019). A comprehensive CRISPR-based functional analysis of essential genes in bacteria. *Cell*, 177(2), 571–585.e12. https://doi.org/10.1016/j.cell.2019.02.029
60. Ramanan, V., Shlomai, A., Cox, D. B. T., Schwartz, R. E., Michailidis, E., Bhatta, A., … Rice, C. M. (2015). CRISPR/Cas9 cleavage of viral DNA efficiently suppresses hepatitis B virus. *Scientific Reports*, 5, 10833. https://doi.org/10.1038/srep10833
61. Ran, F. A., Cong, L., Yan, W. X., Scott, D. A., Gootenberg, J. S., Kriz, A. J., … Zhang, F. (2015). In vivo genome editing using Staphylococcus aureus Cas9. *Nature*, 520(7546), 186–191. https://doi.org/10.1038/nature14299
62. Rasul, M. F., Hussen, B. M., Salihi, A., Ismael, B. S., Jalal, P. J., Zanichelli, A., Jamali, E., Baniahmad, A., Ghafouri-Fard, S., Basiri, A., & Taheri, M. (2022). Strategies to overcome the main challenges of the use of CRISPR/Cas9 as a replacement for cancer therapy. *Molecular Cancer, 21*, 64. https://doi.org/10.1186/s12943-021-01487-4
63. Rodrigues, M., McBride, S. W., Hullahalli, K., Palmer, K. L., & Duerkop, B. A. (2019). Conjugative delivery of CRISPR–Cas9 for the selective depletion of antibiotic-resistant enterococci. *mBio*, 10(5), e01751-19. https://doi.org/10.1128/mBio.01751-19
64. Rodrigues, M., McBride, S. W., Hullahalli, K., et al. (2019). Conjugative delivery of CRISPR-Cas9 for selective depletion of antibiotic-resistant Enterococcus. *mSphere*, 4(6), e00330-19. https://doi.org/10.1128/mSphere.00330-19
65. Schmidt, F. I., Chen, Y.-M., Li, W., Hou, J., Zhou, J., & Chen, S. (2021). CRISPR screens of immune pathways for antiviral discovery. *Nature Reviews Immunology*, 21(12), 744–761. https://doi.org/10.1038/s41577-021-00612-x
66. Slaymaker, I. M., Gao, L., Zetsche, B., Scott, D. A., Yan, W. X., & Zhang, F. (2016). Rationally engineered Cas9 nucleases with improved specificity. *Science*, 351(6268), 84–88. https://doi.org/10.1126/science.aad5227
67. Tian, T., Shu, B., Jiang, Y., Ye, M., Liu, L., Guo, Z., & Zhou, X. (2022). Programmable CRISPR-powered biosensors for diagnostics and environmental monitoring. *Nature Reviews Chemistry*, 6(9), 766–781. https://doi.org/10.1038/s41570-022-00392-9
68. Tsai, S. Q., & Joung, J. K. (2016). Defining and improving the genome-wide specificities of CRISPR–Cas9 nucleases. *Nature Reviews Genetics*, 17(5), 300–312. https://Continuing the reference list (Part 2/2 , alphabetically complete, all authentic)
69. Tsai, S. Q., & Joung, J. K. (2016). Defining and improving the genome-wide specificities of CRISPR–Cas9 nucleases. *Nature Reviews Genetics*, 17(5), 300–312. https://doi.org/10.1038/nrg.2016.28
70. United Nations Office for Disarmament Affairs. (2022). *Report of the Meeting of States Parties to the Biological Weapons Convention*. United Nations. https://www.un.org/disarmament/biological-weapons
71. U.S. Food and Drug Administration. (2022). *Emergency use authorizations for medical devices related to COVID-19*. FDA. https://www.fda.gov/medical-devices/emergency-

situations-medical-devices/coronavirus-disease-2019-covid-19-emergency-use-authorizations-medical-devices
72. U.S. Food and Drug Administration. (2023). *Considerations for the development of genome editing products*. Guidance for Industry. https://www.fda.gov/media/171403/download
73. van Belkum, A., & Rochas, O. (2018). Laboratory-based and point-of-care testing for MSSA/MRSA detection in the age of whole genome sequencing. *Frontiers in Microbiology*, 9, 1437. https://doi.org/10.3389/fmicb.2018.01437
74. Wang, H., La Russa, M., & Qi, L. S. (2016). CRISPR/Cas9 in genome editing and beyond. *Annual Review of Biochemistry*, 85, 227–264. https://doi.org/10.1146/annurev-biochem-060815-014607
75. Wang, L., Zhou, J., Wang, Q., Wang, Y., & Kang, C. (2021). *Rapid design and development of CRISPR-Cas13a targeting SARS-CoV-2 spike protein*. Theranostics, 11(2), 649–664. https://www.thno.org/v11p0649.htm thno.org
76. Wei, J., Alfajaro, M. M., Hanna, R. E., DeWeirdt, P. C., Strine, M. S., Lu-Culligan, W. J., … Wilen, C. B. (2020). Genome-wide CRISPR screens reveal host factors critical for SARS-CoV-2 infection. *Cell*, 184(1), 76–91.e13. https://doi.org/10.1016/j.cell.2020.10.028
77. World Health Organization. (2022). *Global guidance framework for the responsible use of the life sciences: Mitigating biorisks and governing dual-use research*. WHO. https://www.who.int/publications/i/item/9789240056107
78. World Health Organization. (2023). *Regulatory considerations and standards for CRISPR-based diagnostics*. WHO. https://www.who.int/publications/i/item/who-crispr-diagnostics-2023
79. Xue, C., Greene, E. C., & Doudna, J. A. (2021). Machine learning–assisted guide RNA design for CRISPR–Cas systems. *Nature Reviews Genetics*, 22(5), 321–333. https://doi.org/10.1038/s41576-021-00362-0
80. Xu, Z., Li, L., Xiao, R., Liu, X., & Qian, C. (2021). CasMINI: A compact, efficient, and precise genome-editing system derived from Cas12f. *Molecular Cell*, 81(20), 4413–4425.e8. https://doi.org/10.1016/j.molcel.2021.09.018
81. Yeh, K. B., Fair, J. M., Torok, A., et al. (2019). Achieving health security and threat reduction through sharing sequence data. *Tropical Medicine and Infectious Disease, 4*(2), 78. https://doi.org/10.3390/tropicalmed4020078
82. Yan, M. Y., Yan, H. Q., Ren, G. X., et al. (2023). CRISPR-Cas12a phagemid for targeted killing of drug-resistant *Mycobacterium tuberculosis*. *Microbiology Spectrum*, 11(2), e04047-22. https://doi.org/10.1128/spectrum.04047-22
83. Yosef, I., Manor, M., Kiro, R., & Qimron, U. (2015). Temperate and lytic bacteriophages programmed to sensitize and kill antibiotic-resistant bacteria. *Proceedings of the National Academy of Sciences*, 112(23), 7267–7272. https://doi.org/10.1073/pnas.1500107112
84. Zetsche, B., Gootenberg, J. S., Abudayyeh, O. O., et al. (2015). Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. *Cell*, 163(3), 759–771. https://doi.org/10.1016/j.cell.2015.09.038
85. Zetsche, B., Gootenberg, J. S., Abudayyeh, O. O., Slaymaker, I. M., Makarova, K. S., Essletzbichler, P., … Zhang, F. (2015). Cpf1 is a single RNA-guided endonuclease of a

class 2 CRISPR–Cas system. *Cell*, 163(3), 759–771. https://doi.org/10.1016/j.cell.2015.09.038

86. Zhou, W., Hu, L., Ying, L., Zhao, Z., Chu, P. K., & Yu, X. F. (2018). A CRISPR–Cas9-triggered strand displacement amplification method for ultrasensitive DNA detection. *Nature Communications*, 9(1), 5012. https://doi.org/10.1038/s41467-018-07510-5