

Strategies to Minimizing off-Target Effects in CRISPR-CAS9 Genome Editing: A Systematic Literature Review

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Received: June 30, 2026

Revised: July 16, 2026

Accepted: July 25, 2026

Published: July 30, 2026

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Abstract: CRISPR-Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats – Associated Protein 9) is a leading innovation in the field of genome editing due to its high efficiency, flexibility, and accuracy. However, the application of this technology faces challenges regarding off-target effects. Various strategies have been developed to enhance the specificity of CRISPR-Cas9 technology. This study analysis strategies developed over the past decade to minimize off-target effects, based on scientific evidence. A systematic literature review was conducted by examining scientific articles published in international journals indexed in Google Scholar and PubMed. The results indicate that off-target effects can be minimized through a combination of methods, including Cas9 engineering, sgRNA design optimization, the use of base editing, and the application of computational systems for accurate off-target predictions. This study provides an overview of recent developments in improving the safety and precision of genome editing and serves as a scientific reference for future research.

Keywords: CRISPR-Cas9; off-target; genome editing

Introduction

CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats) and its associated proteins have been adapted into a genome-editing tool. Cas9 from *Streptococcus pyogenes* is a blunt-cutting nuclease that targets specific DNA sequences (Fu et al., 2019) and has become a leading technology for precise and efficient gene editing. This technology enables the addition, deletion, or alteration of genetic material within a genome at specific locations (Riaz et al., 2025; Wang, et al., 2025). CRISPR-Cas9 was discovered in bacteria and archaea as an adaptive immune system (Martin et al., 2016; Naeem & Alkhnabashi, 2023; Luo et al., 2024; Liu et al., 2020). Upon viral infection, the system stores information in segments known as CRISPR arrays—consisting of viral genomic DNA segments (spacers) acquired and separated by palindromic sequences. Transcription of the array generates CRISPR-RNA (crRNA) or guide RNA (gRNA) (Naeem & Alkhnabashi, 2023).

CRISPR-Cas9 can induce precise modifications in genomic sequences, though this carries the risk of off-target effects (Wang et al., 2018; Zhang et al., 2023). Such off-target effects can lead to lethal mutations within gene exons or introns, or elsewhere in the genome. Numerous computational tools have been developed to design effective gRNAs based on predictions of on-target activity and potential off-target effects. These tools are used to optimize CRISPR-Cas9 research, employing methods ranging from simple alignment-based approaches to those incorporating target locus sequences and epigenetic data integration. (Naeem & Alkhnabashi, 2023). To recognize a target, Cas9 binds to a short DNA sequence known as the Protospacer Adjacent Motif (PAM) and checks for complementarity with the gRNA. If mismatches and bulges within the gRNA-DNA hybrid—or within the PAM itself—are

tolerated, Cas9 cleavage may occur at sites other than the intended target, resulting in off-target editing (Antho et al., 2022; Lin & Wong, 2018).

Computational tools have been developed to optimize this process, specifically covering off-target identification, on-target efficiency prediction, and the modeling of editing outcomes. By utilizing these methods, researchers can screen and evaluate highly efficient sgRNAs prior to conducting experiments, thereby enhancing design accuracy and increasing the likelihood of experimental success (Du et al., 2026). Consequently, by leveraging existing research data, we analyzed various strategies to minimize off-target effects in CRISPR-Cas9 technology. The findings of this study are expected to serve as a foundation for the development of gene-editing technologies that are more precise, safe, and efficient.

Methods

This study identifies findings from various publications regarding the minimization of off-target effects in CRISPR-Cas9 technology. The research process involved identifying and screening articles based on specific criteria, assessing their suitability, and selecting them for inclusion. The articles were sourced from reputable scientific databases, namely Google Scholar and PubMed. The search was limited to English-language articles published between 2016 and 2026 to reflect the latest developments in CRISPR-Cas9 technology and strategies for minimizing off-target effects. The selected articles consist of original research—featuring experimental, computational, and scientific synthesis data—that is relevant to the study's objectives and available in full-text format. The gathered data are presented in both tabular and narrative forms.

Results and Discussions

Table 1 summarizes the principal strategies employed to minimize off-target effects in CRISPR-Cas9 genome editing.

Table 1. Strategies to Minimizing Off-Target Effects in CRISPR-Cas9

No.	Authors	Title	Method	Results
1.	Han <i>et al.</i> (2023)	Minimizing the off-target frequency of the CRISPR/Cas9 system via zwitterionic polymer conjugation and peptide fusion	chemically conjugating the Cas9 protein with the zwitterionic polymer pCB and testing on-target and off-target efficiency	Off-target editing efficiency in zwitterion-modified CRISPR/Cas9 is reduced by 70% to 90%.
2.	Cao et al. (2016)	An easy and efficient inducible CRISPR/Cas9 platform with improved specificity for multiple gene targeting	Combination of iCas9 and multiplex sgRNA	Enables tight temporal control, multiplex targeting, and a reduction in off-target effects.
3.	Yu et al. (2026)	Guide RNA reprogramming facilitates minimized tracrRNA-dependent off-target and versatile	Formulating optimal gRNA design rules and engineering crRNA variants that do not match the	Developing a reprogrammable tracrRNA to detect mRNA via CRISPRa and

		CRISPR/Cas9 engineering	human or mouse transcriptome, using machine learning via high-throughput gRNA variant screening.	redesigning the scaffold to suppress PAM-independent self-editing by Cas9; subsequently, creating a separately expressed gRNA platform featuring distinct tracrRNA and non-repetitive tandem crRNA components capable of multiplex editing of up to six genes and enhancers in stem cells.
4.	Shen et al. (2018)	Synthetic switch to minimize CRISPR off-target effects by self-restricting Cas9 transcription and translation	Designing a synthetic switch by combining the Cas9/gRNA system and the L7Ae:K-turn system.	Cas9 expression is tightly repressed, resulting in high-efficiency on-target indel mutations while minimizing off-target effects.
5.	Garood et al. (2021)	Analysis of off-target effects in CRISPR-based gene drives in the human malaria mosquito	Detecting off-target mutations <i>in vivo</i> for CRISPR-Cas9-based gene drives at the genome level by combining an identification step using CIRCLE-seq with deep sequencing.	Off-target editing in <i>An. gambiae</i> was successfully identified and predicted.
6.	Tran et al. (2022)	Precise CRISPR-Cas-mediated gene repair with minimal off-target and unintended on-target mutations in human hematopoietic stem cells	"Spacer-nick" involves combining Cas9D10A with a pair of PAM-out sgRNAs spaced more than 200 bp apart.	Gene correction efficiency ranged from 20% to 50%, with minimal NHEJ-mediated on-target mutations. Regarding off-target effects, unintended genetic changes were significantly reduced—or even

				undetectable—in HSPCs.
7.	Pan et al. (2022)	Massively targeted evaluation of therapeutic CRISPR off-targets in cells	SURRO-seq to simultaneously evaluate thousands of therapeutic RGN OTs within cells.	RGN-induced indels were detected in cells using a combination of lentiviral OT capture and deep sequencing. SURRO-seq analysis of 8,150 OTs from 110 therapeutic RGNs successfully identified significant indels at 783 OTs.
8.	Zhao et al. (2024)	Evolved cytidine and adenine base editors with high precision and minimized off-target activity by a continuous directed evolution system in mammalian cells	CDEM is used to engineer the evolution of cytidine deaminase in CBEs with narrower editing windows, as well as adenosine deaminase in ABEmax and ABE8e, independently.	All evolved variants exhibited lower off-target activity at both the DNA and RNA levels. The evolved BE4 variant demonstrated higher product purity.
9.	Coelho et al. (2020)	CRISPR GUARD protects off-target sites from Cas9 nuclease activity using short guide RNAs	CRISPR GUARD for protecting off-target sites, alongside short guide RNAs tested in vitro.	CRISPR GUARD can reduce off-target mutagenesis while maintaining on-target editing efficiency when using Cas9 or base editors.
10.	Zhou et al. (2022)	Eliminating predictable DNA off-target effects of cytosine base editor by using dual guiders including sgRNA and TALE	Cytosine Base Editor (CBE) with a dual-guide system via the combination of CRISPR and Transcription Activator-Like Effector (TALE)	Off-target DNA effects can be minimized.

CRISPR-Cas9 genome editing technology has ushered in a transformative era in the field (Aljabali et al., 2024), offering immense potential for treating both genetic and non-genetic

diseases. Despite this great promise, the technology faces a major challenge: off-target effects (Guo et al., 2023; Zischewski et al., 2017). Various methods have been developed to enhance the efficiency and safety of CRISPR-Cas9-based research, specifically aiming to minimize off-target effects. Imbalanced data presents a common challenge in predicting these effects; consequently, efficient computational techniques are required to address this issue (Zhang & Jiang, 2022).

Han et al. (2023) conducted research utilizing protein-polymer conjugation and protein-peptide fusion technologies to tackle the off-target problem. Following modification, the CRISPR-Cas9 system demonstrated a significant reduction in off-target effects without compromising on-target editing frequency.

Cao et al. (2016) employed shortened guide sequences to minimize the potential for off-target effects. A similar approach was taken by Shen et al. (2018), who successfully minimized off-target effects by shortening gRNA sequences and chemically engineering gRNA to regulate Cas9 expression. This research highlights the importance of attenuating and restricting Cas9 activity to reduce side effects. Garood et al. (2021) also investigated off-target effects by shortening gRNA, conducting *in vivo* analysis of off-target mutations associated with a homing-type gene drive in *Anopheles gambiae*.

Tran et al. (2022), the spacer-nick method was developed for safe and precise gene correction; the use of Cas9n and a pair of sgRNAs demonstrated that this method is highly effective in minimizing off-target effects. Research conducted by Tycko et al. (2019) identified sgRNAs with low specificity as the primary cause of off-target activity. Since Cas9 induces DNA double-strand breaks (DSBs), the presence of numerous off-target sites associated with a given sgRNA can have significant consequences. However, as epigenetic modulation by dCas9 does not result in DSBs, off-target effects in this context are thought to be location-dependent.

In their study, Pan et al. (2022) utilized high-fidelity SpCas9 (HiFi-SpCas9) and demonstrated that the majority of off-target indels were not significantly detected, suggesting its potential for widespread application. Zhao et al. (2024) also conducted research yielding results that indicated lower off-target activity. These findings highlight the importance of more targeted editing, as it not only reduces off-target effects but also enhances editing precision. Efficient single-strand cleavage can offer advantages in genome editing; consequently, identifying and assessing the potential off-target effects of CRISPR-Cas9 nucleases is crucial (Fu et al., 2019).

Coelho et al. (2020) conducted research using the GUARD RNA method, which offers significant potential for preventing off-target mutations. GUIDE-seq (genome-wide unbiased identification of double-stranded breaks enabled by sequencing) enables the efficient and comprehensive identification of homology-independent off-target effects (Chaudhari et al., 2020). GUIDE-seq provides a flexible analysis platform for the identification and annotation of nuclease-induced off-target cleavage sites (Zhu et al., 2017).

Currently, computational analysis plays a vital role in sgRNA design. While machine learning methods utilizing large-scale data have achieved significant success, achieving precise targeting efficiency and sgRNA specificity remains a major challenge (Liu et al., 2019). Research by Yu et al. (2026) offers a solution to mitigate CRISPR off-target effects through the use of machine learning (ML)-designed gRNAs and scaffolds resistant to TDO and self-editing, thereby establishing a framework for CRISPR systems.

On the other hand, results from the study by Zhou et al. 2022 demonstrates that combining CRISPR and TALE can minimize off-target effects, making the approach safe for use in gene therapy. Constructing TALE vectors poses a significant challenge due to their highly repetitive sequences, necessitating simple, rapid, and efficient methods for assembling TALE repeat units.

By utilizing efficient and precise CBEs—combined with CRISPR and TALE to minimize predictable DNA off-target effects—gene therapy and the generation of genetically engineered organisms become safer practices (Zhou et al., 2022).

Conclusion

The greatest challenge facing CRISPR-Cas9 genome editing technology is the occurrence of off-target effects. Genetic engineering efforts continue to focus on mitigating these effects and enhancing therapeutic efficiency. Machine learning methods applied to sgRNA design have proven capable of minimizing off-target effects, a result mirrored by the use of combined experimental approaches. Research indicates that shortening the gRNA sequence can significantly reduce the likelihood of off-target effects, demonstrating that a more restricted editing range enhances precision.

This study reveals that improving the specificity of CRISPR-Cas9 technology relies not only on innovations in Cas9 proteins but also on integrating genetic engineering with mutation detection technologies. This approach lays the foundation for developing genome editing technologies that are safer, more precise, and ready for widespread application across various healthcare sectors.

Acknowledgments

The author thanks all those who supported of this study.

Conflicts of Interest

The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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