Proceedings of the 6th International Conference on Biological Engineering and Medical Science
DOI: 10.54254/2753-8818/2026.35705

How to Mitigate CRISPR-Cas9 Off-Target Effects: Evaluation of Current Detection, Prediction, and Mitigation Strategies

Yanbing Xu

The High School Affiliated to Renmin University of China International Curriculum Center, Beijing, China
Helen.xu0420@outlook.com

Abstract. One of the most efficient and powerful genome-editing instruments, CRISPR-Cas9 represents a high promise in treating disease and examining the genome. Nevertheless, its clinical translation is limited by off-target effects that cause harmful genomic mutations and potentially fatal safety issues. This review provides a thorough overview of the current state of knowledge on off-target causes, detection and prediction methods, and mitigation strategies. MAIN REASONSThese include mismatch tolerance of Cas9-gRNA and sustained expression associated with some delivery methods. Finally, the detection and prediction tools based on representative approaches such as GUIDE-seq, CIRCLE-seq, Cas-OFFinder and DeepCRISPR are evaluated and their advantages and limitations are listed. The mitigation strategies such as sgRNA engineering, Cas9 protein engineering and transient delivery systems are also discussed. The study argues that the data mentioned above only measures an important first step, and that the realization of clinically relevant detection models that reliably mimic in vivo conditions is critical to screening safety. This review integrates these dimensions, so as to offer a clear framework for controlling when, where and how. Integrating these dimensions allows this review to provide a clear framework for controlling when, where and how CRISPR-Cas9-mediated alterations occur afford systemic implementation strategies that improve the safety of CRISPR-Cas9 applications.

Keywords: CRISPR-Cas9, off-target effects, gene editing, mitigation strategies, delivery systems

1. Introduction

CRISPR-Cas9 is a highly efficient genome editing system. This broadened the possibility of treating diseases and doing genome studies. The molecular mechanism of the technology is that the Cas9 protein with bound tracrRNA and crRNA forms a complex. The complex makes cuts in the double-stranded DNA at particular sites as directed by these RNA molecules [1]. However, the biggest challenge for its translation into clinic is the off-target effect, which means that the system digs at

DOI: 10.54254/2753-8818/2026.35705

wrong sites in genome. OmniCure makes it to these off-target events that may lead to harmful genomic mutations and pose significant safety challenges.

To tackle this problem, work in the field mainly revolves around three topics: understanding off-target causes& thriving on accurate detections and mitigation; These domains have seen a tremendous upticks in the last couple of years. Indeed, a deeper understanding of off-target causes was gained with many important determinants identified such as the mismatch tolerance between sgRNA and DSB in DNA sequence and genomic binding site features [2, 3]. There are also a number of novel technologies that have been developed to improve off-target site screening in detection and prediction. In addition, various technical solutions have been identified in mitigation strategies, such as sgRNA optimisation, engineered Cas9 proteins and delivery systems [4]. This systematic approach has paved a path for tackling the off-target issue reasonably well.

However, because new technologies are developing so quickly, reviews that do exist cover only a particular perspective. However, an integrated evaluation of these aspects compared to recent solutions is still unavailable. Hence, it is impossible to get a direct overall picture of the potential and inter-relationship/efficacy of various technical strategies by researchers in an immediate environment.

Against this of background, this dissertation will try to provide a relatively systematic overview about the current studies on CRIPSR-Cas9 off-target effects. In this review, the first subject that will be covered is the molecular mechanism of CRISPR-Cas9 system, which allows discussion about the off-target effects. Secondly, it will try to be uniform in arranging the existing on- and off-target effect detection methods by defining them based on their setting. In addition, the different mechanisms that cause off-target effects will be discussed. Mainstream mitigation will be collated, evaluated and finally assessed for effectiveness/weaknesses.

Thus, this review not only summarizes the recent advances in different fields of research but also provides an overarching analytical framework linking mechanisms, detection and prediction methods along with mitigation approaches of the off-target effects. This paper makes this linkage by systematically comparing the fundamental principles, strengths and weaknesses of these dimensions. This type of integration is quite important to the discipline, as it seeks to bring together disparate research works and provide a framework with which future plans for genome editing could operate in a more rational way.

This is a literature review dissertation in which the literature is summarised, contrasted and interpreted. It tries to paint a definite picture of where research about CRISPR-Cas9 off-target effects currently stands and where it is headed in the future.

2. Methodology

The dissertation research approach is largely based on a literature review, relying solely on secondary sources. This methodology has helped to accumulate a great deal of information regarding CRISPR-Cas9 gene editing, specifically the cause of off-target effects, analysis of different detection and prediction methods, as well as mitigation strategies.

The reviews and articles included in the dissertation were from reputable scholarly databases and resources, such as PubMed, Google Scholar, and the websites of journals like Nature and Science.

To ensure the accuracy and comprehensiveness of the research, various keywords were applied on different parts, including mechanism-related terms; cause terms like "mismatch tolerance","delivery methods"; detection and prediction technique terms like "CIRCLE-seq","DeepCRISPR"; and mitigation strategy terms like"sgRNA design" and "optimizing Cas9 protein".

DOI: 10.54254/2753-8818/2026.35705

When screening and selecting the reviews cited, the CRAAP criteria were strictly followed. The studies within the past 5 years, particularly in fast-developing areas such as detection and mitigation methods, were sought at first. Some earlier but foundational research in the CRISPR-Cas9 domain was also included because these studies provide valuable and inspiring theories. Moreover, the abstracts and core paragraphs of the review are first to see whether the material was applicable to the dissertation and could easily be used to support the arguments. Finally, it is ensured that only articles with professional expression and objective data were selected for authority and accuracy.

In conclusion, the reliance on secondary literature ensures the validation and comparison of multiple findings, thereby identifying the common view and research gaps. Moreover, comparing and analyzing conclusions from various experimental backgrounds is highly helpful for this dissertation to surpass the scope of a single research project.

3. Literature review

3.1. Mechanism

By and large, the research method for a dissertation revolves around a review of available literature with reference to secondary sources only. Such methodology has contributed to the accumulation of vast amount of information relating to CRISPR-Cas9 gene editing — including the causes of off-target effects, analyses of various detection and prediction methods, and mitigation strategies.

The review and journal articles included in the dissertation were obtained from reliable academic databases and resources like PubMed, Google Scholar, and the websites for journals such as Nature and Science.

Various keywords related to mechanisms, causes (mismatch tolerance, delivery methods), detection and prediction techniques (CIRCLE-seq, DeepCRISPR), and mitigation strategies (sgRNA design & optimizing Cas9 protein) across the different parts.

The CRAAP criteria were strictly followed when screening and selecting the reviews cited. The first focus was on studies from the last five years and on rapid developing fields like detection and mitigation methods. We also included some earlier, but more foundational work in the CRISPR-Cas9 space [5], since these works provide rich theories that can inspire later studies. Also, the first search was the abstracts and core paragraphs of the review to see whether it contained material that is applicable to this blast dissertation had a low effort to reuse it for an argument. Lastly, the articles selected for authority and accuracy would consist only of professional expression and non-subjective evidence.

Finally, they trust in secondary literature to confirm and compare more results apart from their own literature, for similarities or differences that may extract the general concept of the viewpoint and areas of research. Furthermore, the comparison and analysis of findings from different experimental contexts is particularly well suited to this dissertation so as to be greater than a single research project.

3.2. Cause

The error that commonly occurs during the gene editing process is the off-target effect, which raises significant concerns regarding the clinical use of the CRISPR-Cas9 technique. A variety of reasons have been considered as the causes of off-target effects. Among them, the Cas9 mismatch tolerance and the choice of delivery methods are the most significant.

DOI: 10.54254/2753-8818/2026.35705

It is worth noting that the CRISPR system can accommodate one or two mismatches in the target sequence, depending on their location. More concerning is the fact that even off-target sites with up to five mismatches may undergo mutation at a rate equal to or greater than that of the intended target site [6]. This was later proved in studies using SpCas9, which also found the mismatch tolerance between guide RNA and target DNA at various positions, leading to off-target effects [7].

Off-target effects in CRISPR-Cas9 also depend on delivery methods, which primarily involve viral and non-viral vectors, as well as physical approaches. Viral vectors, such as AAV, are often chosen for in vivo applications due to their delivery efficiency. However, they have limited packaging capacity, and their prolonged expression of CRISPR components can increase the likelihood of off-target editing. Non-viral vectors, such as plasmids carrying Cas9 and sgRNA, are more commonly used in vitro. Yet plasmid DNA may integrate into the host genome, leading to unintended off-target modifications [8].

3.3. Detection: GUIDE-seq and CIRCLE-seq

GUIDE-seq is a non-biased and reliable cell-based approach for CRISPR-Cas9 off-target detection. The technique innovatively transfects cells with dsODN, a short, synthetic double-stranded DNA molecule, into cells. Its key function is to mark the break sites caused by Cas9. This approach can mark all break sites precisely, making it rather easy to predict and observe off-target sites [9].

Another approach, which is CIRCLE-seq, is a highly sensitive in vitro method for comprehensively identifying CRISPR-Cas9 off-target effects. This technique can innovatively circularize genomic DNA and externally perform Cas9 cleavage to enable precise identification of off-target sites without interference from chromatin context [1].

3.4. Prediction: Cas-OFFinder and DeepCRISPR

Cas-OFFinder uses a sequence alignment algorithm based on OpenCL. Its core function is to efficiently search the entire genome for potential off-target sites that are similar to a given gRNA sequence and match the PAM sequence defined by the user [10]. Cas-OFFinder uses GPU acceleration to achieve high speed. However, it only identifies potential off-target sites and is not designed to predict the on-target knockout efficacy. It also does not consider whether the gene is accessible in a chromatin context or affected by epigenetic modifications.

DeepCRISPR is a predictive deep learning model for CRISPR gene editing designs. It uses a two-stage training approach: first, it learns the general features independently from large amounts of sgRNA sequences, then it adjusts the model using precisely labeled experimental data [11]. DeepCRISPR can predict both gRNA editing efficiency and genome-wide off-target effects. It also has the ability to automatically find non-linear correlations between sequences and editing outcomes. However, the model demands large amounts of high-quality labeled data and computational resources, which are often inaccurate and insufficient.

3.5. Mitigation

In the continuous evolution of the CRISPR-Cas9 system, it has been demonstrated that the modification of sgRNA is essential in minimizing off-target effects. The main optimization strategies can be broadly categorized into two approaches: structural optimization and chemical modification of sgRNA.

DOI: 10.54254/2753-8818/2026.35705

The sgRNA structural engineering is achieved by shortening the length of the sgRNA or truncating its length. This approach may significantly increase the specificity of the sgRNA, therefore reducing off-targets [12, 7]. However, the optimal sgRNA length depends on the target site and needs to be determined experimentally [11].

Apart from structural optimization strategies, chemical modification represents another cutting-edge approach for designing sgRNA. As an example, adding 2'-O-methyl or LNA at certain sites can be used to stabilize the sgRNA and slow down the reaction kinetics of the Cas9 protein [12]. The primary advantage of chemical modification is its significant improvement in editing efficiency and stability. However, this technique is only possible currently in synthetic RNA systems.

As an important component of the CRISPR-Cas9 system, the Cas9 protein has not only brought innovation in gene editing but has also been a primary factor in off-target effects, indicating the importance of Cas9 protein optimization.

A significant type of approach to mitigate off-target effects is engineering the Cas9 protein. One of the strategies is the engineering of the catalytic domain to modify its activity. By creating mutations in the catalytic residues of Cas9 nuclease, such as HNH and RuvC, it can be converted into a nickase [13].

Another approach is directed evolution. It is important to note that variant iGeoCas9 was developed based on directed evolution to increase the thermostability and stability. Its editing efficiency is surprisingly better than that of the unmodified SpCas9 when it is delivered by using lipid nanoparticles (LNPs) [11].

When applying CRISPR-Cas9 gene editing, the delivery methods chosen can have an impact on the persistence of CRISPR components, which may affect the potential occurrence of off-target effects.

Transient delivery is facilitated by physical delivery methods like electroporation and non-viral delivery methods, including lipid nanoparticles (LNP). Such a strategy allows the editing components to degrade quickly, thereby restricting off-target threats [12]. Specifically, the use of the ribonucleoprotein (RNP) complex through LNP can greatly lower the possibility of off-target effects because the intracellular time of the complex is rather brief [13].

4. Discussion

In the literature review, by synthesizing existing studies, this review systematically introduced four detection and prediction strategies for off-target effects and three categories of mitigation approaches. However, it has remained at the level of describing the basic mechanisms and characteristics of these methods. In the following section, this section will focus on analyzing the advantages and disadvantages of each approach, as well as their applicable scenarios, while offering a critical thinking.

The dissertation first addresses the major causes of off-target effects. Through secondary research, it was discovered that the tolerance of Cas9-gRNA complex may lead to mismatches between guide RNA and target DNA, thereby causing off-target effects [6, 9]. Moreover, the delivery method is also an important factor. Viral vectors such as AAV and non-viral vectors such as plasmid transfection can result in long-term expression, thus increasing the risk of off-target effects [8]. Therefore, the off-target potential is directly related to the internal factors, including tolerance to mismatches in sequence matching, and external factors, including exposure time, which depends on the editing and delivery tools selected.

In summary, the two major causes of off-target effects are the primary focus of this dissertation. The safety risks due to mismatch tolerance require detection methods with high sensitivity.

DOI: 10.54254/2753-8818/2026.35705

Meanwhile, the prolonged expression time in the cellular environment due to the delivery methods chosen requires a detection method that marks most DNA break sites to predict and detect off-target sites. In addition, the mitigation methods are directly linked to the two major causes. The precision in sequence recognition can be enhanced by engineering sgRNA and introducing Cas9 mutations. Moreover, selecting delivery methods to regulate the expression duration of CRISPR components is another option. Hence, this section has illustrated the core requirements in selecting both detection methods and mitigation approaches, establishing a direction for further subsequent passages.

GUIDE-seq and CIRCLE-seq have been chosen because of their sensitivity and representativeness at cellular and in vitro levels.

GUIDE-seq is a genome-wide, cell-based approach that uses transfected dsODN to mark Cas9 cleavage sites in living cells [10]. GUIDE-seq has two major advantages: first, it has high sensitivity in living cells, which allows GUIDE-seq to work in the actual physiological environment. Second, it can be applied in a wide range of cell types. Therefore, its results are more accurate and highly practical. However, GUIDE-seq relies on the efficient delivery of Cas9 and the dsODN marker, making it challenging in cells that are difficult to transfect. For this reason, GUIDE-seq is only suitable for pre-clinical studies to screen and optimize sgRNAs and Cas9 variants.

While cell-based methods such as GUIDE-seq can detect off-target effects in a cellular environment, their efficiency can be reduced. Another detection approach is CIRCLE-seq. The key advantage is that it is not dependent on living cells or transfection. Purified DNA ensures high sensitivity, low interference, and detection of low-frequency potential off-targets compared to GUIDE-seq. Moreover, it is scalable, fast, and reproducible. However, it does not mimic the intracellular nuclear environment, so some detected sites may not occur in real cells. Due to these potential false positives, the results require further verification in vivo environments. These false-positive sites produced by in vitro detections can exaggerate the frequency of off-target activities. This may not only mask genuine off-target events, but also interfere with preclinical safety evaluations, and even lead to misjudgment of potential medicines.

Overall, the two approaches are complementary. CIRCLE-seq is more appropriate for preliminary comprehensive screening and high-risk sites' discovery, especially for cells that are hard to transfect, or in events whose frequency is low. GUIDE-seq is more suitable for further validation and studies of physiological relevance, especially when assessing cell-type specificity or chromatin state. Still, when it comes to clinical use, which is the major objective of this dissertation, GUIDE-seq would be a better choice. Since it is more useful in living cells by allowing direct detection, it enables personalized assessment for individual patient cells, satisfying the requirements of translational research.

Among computational tools, Cas-OFFinder and Deep CRISPR are representative and widely used. Cas-OFFinder is a traditional alignment-based tool for high-throughput scanning. The major merit is its high computation speed and efficiency due to the GPU-accelerated alignment, making it suitable for rapid preliminary screening. Moreover, its algorithm is transparent, and the results can be explained and understood. However, its capability is limited: it only identifies sites with high similarity in their sequences and ignores epigenetic factors like chromatin accessibility, as it only relies on sequence matching. As a result, the accuracy is limited, and false positives may be produced in regions of high similarity.

On the other hand, DeepCRISPR is a modern deep learning model that combines sequence and epigenetic information. Its key advantage is to predict the efficacy of on-target editing and genome-wide off-target effects with higher accuracy and generalizability across cell types. But it highly relies on the quality of data used for model training, and the performance declines when the data is

DOI: 10.54254/2753-8818/2026.35705

insufficient. Moreover, as a deep learning model, it requires a large amount of computational resources, and the process to generate results lacks interpretability, which limits its application.

All in all, DeepCRISPR will be more suitable for clinical applications than Cas-OFFinder. The reason behind this is that clinical applications require high accuracy and safety, while DeepCRISPR considers both DNA sequences and epigenetic factors, including chromatin accessibility. Therefore, its predictions are closer to the actual in vivo outcomes. In contrast, Cas-OFFinder only looks at the similarity of sequences and may produce false positives, making it more appropriate for preliminary screening in labs rather than making clinical decisions. The main mitigation strategies and their applicable scenarios are summarized in Table 1.

4.1. Mitigation

Of these, structural optimization and chemical modification are the two strategies that have most often been mentioned for sgRNA engineering. The basis of the structural optimization is that it would reduce sgRNA from approximately 100 nucleotides to a version of a length of 17–18 nucleotides. By confining interaction space, this allows for a tighter binding and thus more accurate pairing between the gRNA seed region and target DNA. In conclusion, structural optimization is a relatively simple and economical approach that can be implemented in high throughput screening. However, this can be very sequence-dependent and is largely used for initial screening and simple investigations [11]. Chemical modification is different because it provides the ability to introduce precise mutations in either the end or seed region of sgRNA, and sometimes incorporates locked nucleic acids (LNA) into non-seed regions. The modifications consisted of 2â€²-O-methyl changes for ribose portions of the sgRNA, which help protect it from degradation from endogenous nucleases in cells and extend its intracellular half-life. On the other hand, LNA stabilizes RNA structure, allowing sgRNA to more specifically hybridize to target DNA and reduce off-target binding. Remarkably, they were inserted without impairing the sgRNA to direct its target recognition sequence as a result the guiding ability of sgRNA is not compromised. In short, sgRNA may be stabilized and made to bind more specifically by chemical modification, leading to an increased editing efficiency and a decreased off-target effect. However, there are still some disadvantages such as its complex and expensive process. In addition, it must be incorporated during RNA synthesis rather than made by the cell itself. Thus, this is now limited to synthetic RNA systems and is still difficult for in vivo expression [12].

4.2. Cas9

The most typical subtypes are nick variants, high-fidelity variants, and directed evolution variants of Cas9 protein engineering. Another possibility is to inactivate one catalytic site; this creates a nickase that can only cut one strand of the double helix. Nonetheless, nickases can be utilized for high-safety applications although they still afford lower editing efficiencies [2]. Mutants of Cas9 rationally designed to have high-fidelity variants include eSpCas9. The variants reduce non-specific binding to off-target sites by changing the DNA binding domain but preserve on-target target-binding efficiency as well as cutting. This allows for them to retain a significant level of editing efficiency while enhancing specificity a great deal, which when combined makes them prime candidates to go from laboratory to preclinical use [7]. The variants of Cas9 able to direct its own evolution are generated by random mutation of the cas9 gene and then screened in high-throughput for novel properties. Firstly, some of your gene editibles possess a high thermal stability and also they are compatible to be carried by LNP delivering vectors as e.g. iGeoCas9 was one of the examples which

DOI: 10.54254/2753-8818/2026.35705

were showing these qualities. But, this is a long development cycle and the variants behave differently in a range of conditions. They are more suitable for specific delivery platforms at this time, however clinical use is still in the preliminary phases [4]. Furthermore, rounding up the tremendous advantages observed in vitro by variants created through random mutations are uncertainties when these variants are put to a true test in vivo. It can elicit unpredictable immune responses, so it needs to be validated comprehensively and rigorously before it is applicable clinically.

4.3. Delivery

In addition to modifying the editing tools themselves, another approach to limit the off-target effects is to regulate the expression duration of CRISPR components through appropriate delivery methods. This approach controls how long the editing tools remain active inside cells, which has a direct impact on off-target effects. Viral vector delivery and transient delivery are the two major strategies. Viral vector delivery primarily uses adeno-associated virus (AAV), while electroporation or lipid nanoparticles (LNP) are primarily used in transient delivery systems to deliver pre-assembled complexes of Cas9-sgRNA ribonucleoprotein (RNP) [2, 7]. AAV vectors and other viral vectors deliver DNA sequences that encode the Cas9 protein and sgRNA into the cells, causing long-term expression of the editing components. This allows them to treat genetic diseases that require ongoing editing, but increases the risk of off-target effects. Therefore, this delivery system is more suitable for applications that require long-term expression and in which the off-target effects are considered acceptable. In comparison, the application of transient delivery is proposed to introduce pre-assembled RNP complexes using the techniques of electroporation or LNP.

These RNP complexes can be quickly degraded in cells and shorten the active window of the editing tools, which essentially reduces the chances of off-target cleavage. Moreover, this approach does not involve the risk of integrating plasmid DNA into the host genome, making it very useful for off-target control. In conclusion, transient delivery methods suit well for both in vitro cell therapies and short-term in vivo editing, demonstrating their practicability. In addition, they can significantly reduce the risk of unpredictable off-target effects and genomic integration caused by prolonged expression, thereby meeting regulators' requirements for gene therapy safety and offering an advantage in the clinical approval process.

In conclusion, the detection and prediction strategies, along with mitigation approaches discussed above, each have their own strengths and limitations. However, the majority of current research today is conducted in vitro or through computational simulations. The internal environment of the human body is particularly complex, and unexpected mutations, immune rejection, or other unpredictable problems may occur. Hence, future studies should prioritize the application of more physiologically relevant models. Meanwhile, animal studies and small-scale human trials should be used before these approaches can be widely applied in patients.

Table 1. Comparison of mitigation strategies and their applicable scenarios

Strategy Category	Specific Approach	Advantages	Limitations	Suitable Applications
sgRNA Engineering	Structural Optimization (Truncated sgRNA)	Low cost, easy to implement, suitable for high-throughput screening.	Effectiveness is sequence-dependent; limited specificity improvement.	Preliminary screening, basic research.

DOI: 10.54254/2753-8818/2026.35705

Table 1. (continued)

	Chemical Modification (e.g., 2′-O-methyl, LNA)	Enhances sgRNA stability and binding specificity; reduces off-target effects without altering target recognition.	Complex and expensive synthesis; currently limited to synthetic RNA systems; challenging for in vivo expression.	Synthetic RNA-based systems, in vitro experiments.
Cas9 Protein Engineering	Nickase Variants	Greatly reduces off-target risk by generating single-strand nicks; high safety.	Often exhibits lower editing efficiency.	High-safety applications, initial gene-editing screening.
	High-Fidelity Variants (e.g., eSpCas9)	Maintains high on-target editing efficiency while significantly improving specificity.	Some variants may still show reduced activity for certain targets.	Laboratory to preclinical studies, applications that require both efficiency and specificity.
	Directed-Evolution Variants (e.g., iGeoCas9)	Exhibits unique properties such as thermal stability and compatibility with LNP delivery.	Long development cycle; performance is context-dependent; clinical translation still in early stages.	Specific delivery platforms, research-stage investigations
Delivery Strategy Optimization	Viral Vectors (e.g., AAV)	Enables long-term expression, suitable for diseases requiring sustained editing.	Prolonged expression increases off-target risk.	Therapeutic scenarios where persistent editing is necessary and acceptable off-target risk.
	Transient Delivery (RNP via LNP/Electroporation)	Shortens editing-tool activity window; reduces off-target opportunity; avoids genomic integration risks.	Editing is transient, unsuitable for applications needing long-lasting expression.	In vitro cell therapies, short-term in vivo editing, suitable for clinical use.

5. Conclusion

This paper provides a critical analysis of the off-target effects caused by CRISPR-Cas9, from which a holistic content framework is established with causes and mechanisms underlying the off-target effects as well as methods detecting and predicting them, in addition to strategy for their mitigation. Abstract Significant progress has been made to understand how a CRISPR–Cas9 genome editing system works at the level of DNA, RNA and protein. To address this, various detection and prediction methods such as GUIDE-seq, CIRCLE-seq, Cas-OFFinder and DeepCRISPR have been developed to accurately identify off-targets. In addition, use sgRNA engineering, Cas9 protein optimization and temporary delivery systems to improve data efficiency. These findings inform directly the purpose of this dissertation, namely assessing the measures to mitigate CRISPR-Cas9 safety.

This study represents a contribution to the field in an attempt to unify disparate results into a systematic investigation of strategies for detection, prediction and mitigation comparing multiple

DOI: 10.54254/2753-8818/2026.35705

experimental and clinical conditions. However, this dissertation may help to fill in the gap between mechanistic theory and strategic targeting selection, thus providing intermediate researchers working towards CRISPR-Cas9 safety optimization with a resource. This review contends that while in vitro approaches are critical and relevant for initial screening and mechanistic understanding, they are highly restrictive for clinical safety. Complex physical conditions in real (in vivo) environments may greatly influence off-target patterns of unmatched phenomena, not replicable or predictable from simplified lab models. This is why, clinical safety now requires definitive in vitro validation. These encourage the development of detection models that are more similar to in vivo settings and delivery systems with durable editing while minimizing off-target effects. In addition, establishment of standard evaluation criteria for different forms of Cas9 and cell types will be important to improve the translation reproducibility.

References

[1] Jinek, M. et al. (2012). A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science, 337*(6096), 816–821. https://doi.org/10.1126/science.1225829
[2] Asmamaw, M. & Zawdie, B. (2021). Mechanism and applications of CRISPR/Cas-9-mediated genome editing. *Biologics, 15*, 353–361. https://doi.org/10.2147/BTT.S326422
[3] Bae, S., Park, J. & Kim, J. (2014). Cas-OFFinder: A fast and versatile algorithm that searches for potential off-target sites of Cas9 RNA-guided endonucleases. *Bioinformatics, 30*(10), 1473–1475. https://doi.org/10.1093/bioinformatics/btu048
[4] Chen, K. et al. (2025). Lung and liver editing by lipid nanoparticle delivery of a stable CRISPR-Cas9 ribonucleoprotein. *Nature Biotechnology, 43*(9), 1445–1457. https://doi.org/10.1038/s41587-024-02437-3
[5] Chuai, G. et al. (2018). DeepCRISPR: Optimized CRISPR guide RNA design by deep learning. *Genome Biology, 19*(1), 80. https://doi.org/10.1186/s13059-018-1459-4
[6] Fu, Y. et al. (2013). High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. *Nature Biotechnology, 31*(9), 822–826. https://doi.org/10.1038/nbt.2623
[7] Guo, C. et al. (2023). Off-target effects in CRISPR/Cas9 gene editing. *Frontiers in Bioengineering and Biotechnology, 11*, 1143157. https://doi.org/10.3389/fbioe.2023.1143157
[8] Horodecka, K. & Düchler, M. (2021). CRISPR/Cas9: Principle, applications, and delivery through extracellular vesicles. *International Journal of Molecular Sciences, 22*(11), 6072. https://doi.org/10.3390/ijms22116072
[9] Hsu, P. et al. (2013). DNA targeting specificity of RNA-guided Cas9 nucleases. *Nature Biotechnology, 31*(9), 827–832. https://doi.org/10.1038/nbt.2647
[10] Li, Z. et al. (2023). Protocol for editing fibroblasts with in vitro transcribed Cas9 mRNA and profile off-target editing by optimized GUIDE-seq. *STAR Protocols, 4*(4), 102662. https://doi.org/10.1016/j.xpro.2023.102662
[11] Matson, A. et al. (2019). Optimizing sgRNA length to improve target specificity and efficiency for the GGTA1 gene using the CRISPR/Cas9 gene editing system. *PLoS ONE, 14*(12), e0226107. https://doi.org/10.1371/journal.pone.0226107
[12] Ryan, D. et al. (2018). Improving CRISPR-Cas specificity with chemical modifications in single-guide RNAs. *Nucleic Acids Research, 46*(2), 792–803. https://doi.org/10.1093/nar/gkx1199
[13] Tsai, S. et al. (2017). CIRCLE-seq: A highly sensitive in vitro screen for genome-wide CRISPR-Cas9 nuclease off-targets. *Nature Methods, 14*(6), 607–614. https://doi.org/10.1038/nmeth.4278