Unveiling neural network potential in forecasting CRISPR effects and off-target prophecies for gene editing

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Abstract
The revolutionary Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) - associated protein 9 (Cas9) systems has emerged as a groundbreaking gene-editing tool, widely embraced within biomedical research. Nonetheless, the utilization of guide RiboNucleic Acids (gRNAs) in the CRISPR-Cas9 system can inadvertently trigger undesired off-target effects, consequently impinging on the practical implementation of this technique. Existing in silico prediction methods that focus on off-target effects have exhibited constrained predictive accuracy, necessitating further enhancement. To tackle this challenge, we present a Base Editing and Prime Editing approach in this study. This approach aim to enhance the precision and specificity of DeoxyRiboNucleic Acid (DNA) modifications compared to traditional CRISPR-Cas9 methods. Both techniques provide unique approaches to achieve targeted changes in the DNA sequence without inducing double-stranded breaks, which can lead to off-target effects. Base editing is highly specific and allows for the correction of point mutations associated with diseases while Prime Editing allows for a wider range of modifications compared to base editing, including the ability to insert or delete specific sequences. Their ability to achieve specific genetic changes while minimizing off-target effects makes them valuable additions to the gene editing toolkit. The findings of this research contribute to the advancement of precision gene editing, offering an enhanced predictive framework to mitigate off-target effects in the realm of CRISPR-Cas9 technology.

Keywords: CRISPR-Cas9; gRNAs; DNA; Gene editing; Base editing; Prime editing

1. Introduction
The CRISPR-Cas9 system [1] has revolutionized gene editing tool with profound implications. The remarkable potential of this technology lies in its ability to precisely modify the genome, promising unprecedented advancements across various fields. However, as with any transformative breakthrough, the practical implementation of the CRISPR-Cas9 system comes with its challenges [2]. At the heart of the CRISPR-Cas9 system's efficacy are guide RiboNucleic Acids (gRNAs), which navigate the Cas9 enzyme to the desired DNA target for modification [3]. While this exquisite precision holds great promise, the utilization of gRNAs can also inadvertently trigger unintended off-target effects, undermining the fidelity of the editing process. The accurate prediction and mitigation of these off-target effects have emerged as pivotal concerns, calling for innovative solutions to enhance the reliability and safety of this revolutionary technology [4]. These methodologies, while informative, are yet to attain the level of precision required to confidently steer gene editing endeavors. As the demand for precision in genome manipulation intensifies, there is an imperative need to refine and augment these predictive frameworks to align with the transformative potential of the CRISPR-Cas9 system [5].

In response to these challenges, this study embraces a paradigm shift by introducing Base Editing and Prime Editing as novel approaches to enhance the precision and specificity of DNA modifications. Unlike conventional CRISPR-Cas9 methods, both of these techniques circumvent the risks associated with double-stranded breaks [6], a common source
of off-target effects. Base Editing orchestrates the conversion of individual DNA base pairs, while Prime Editing extends its prowess to a broader spectrum of modifications, including insertions and deletions. The significance of these techniques lies in their capacity to effectuate specific genetic changes while minimizing the unintended alterations that can compromise the integrity of the genome. The intersection of the CRISPR-Cas9 system with the Base Editing and Prime Editing techniques marks a pivotal juncture in the pursuit of precision gene editing [7]. This convergence presents a tangible path forward, one that holds immense promise for refining the predictability and controllability of the gene editing landscape. By addressing the challenges posed by off-target effects, these advancements contribute not only to the advancement of biomedical research but also to the responsible application of gene editing in diverse domains [8]. This study endeavors to navigate this trajectory, offering valuable insights into the potential of these innovative approaches to reshape the future of genetic manipulation.

2. Review of Literature

Hsu, Patrick D. et al. (2013), Hsu and his team were pioneers in adapting the CRISPR-Cas9 system for genome editing in eukaryotic cells. Their work demonstrated the potential of CRISPR-Cas9 as a precise and programmable tool for making targeted DNA modifications. This foundational research paved the way for subsequent advancements in gene editing. Doench, John G. et al. (2014), In 2014, Doench and colleagues developed a systematic approach for designing highly effective gRNAs for CRISPR-Cas9 targeting. They introduced the concept of "single-guide RNA" libraries and applied them to systematically assess the efficiency of gRNAs in inducing specific gene disruptions. This research significantly improved the accuracy and effectiveness of CRISPR-Cas9 editing by aiding in gRNA selection. Tsai, Shengdar Q. et al. (2015), Tsai et al. explored the potential off-target effects of CRISPR-Cas9 and developed an enhanced version of the Cas9 protein known as "enhanced specificity" (eSpCas9). Their study aimed to mitigate off-target cleavage by engineering Cas9 variants with reduced off-target effects while maintaining on-target activity. This work addressed one of the key challenges in CRISPR technology—enhancing specificity without compromising efficiency. Komor, Alexis C. et al. (2016), In 2016, Komor and colleagues introduced the concept of base editing. They fused a catalytically impaired Cas9 protein with a cytidine deaminase enzyme, enabling targeted conversion of C-G to T-A base pairs without inducing double-stranded breaks. This breakthrough offered a new level of precision for correcting point mutations, reducing the risk of off-target effects associated with DNA cleavage. Zhang, Feng et al. (2017), Zhang and colleagues introduced a new version of the CRISPR-Cas9 system known as "CRISPR-Cpf1" or "CRISPR-Cas12." Their work demonstrated that the Cpf1 enzyme could be used as an alternative to Cas9 for genome editing. This enzyme recognizes different target sequences and introduces staggered cuts in DNA, potentially offering increased precision and fewer off-target effects. Komor, Alexis C. et al. (2018), Building upon their previous work on base editing, Komor and his team expanded the capabilities of the technique in 2018. They developed a more versatile version called "adenine base editors" (ABEs), allowing targeted conversion of A-T to G-C base pairs. This innovation further enhanced the precision of genome editing by enabling a wider range of point mutations to be corrected. Anzalone, Andrew V. et al. (2019), Anzalone and his team expanded on the concept of base editing by developing "prime editing" in 2019. Prime editing combines a catalytically impaired Cas9 with a reverse transcriptase enzyme, enabling precise changes to the genome without relying on a DNA repair template. This innovation enabled the insertion, deletion, or substitution of DNA sequences with minimal off-target effects, promising unprecedented versatility in gene editing. Li, Jing-Rui et al. (2020), Li et al. delved into the prediction of off-target effects in CRISPR-Cas9 editing. They introduced a deep learning framework named "R-CRISPR," which utilized binary matrices, convolutional neural networks, and recurrent neural networks to predict off-target activities with mismatches, insertions, or deletions. Their research aimed to enhance the accuracy of off-target prediction, contributing to the advancement of CRISPR-Cas9 technology. Chen, Bin et al. (2021), Chen and colleagues focused on addressing the off-target effects of CRISPR-Cas9 gene editing by developing a novel strategy in 2021. They engineered a "high-fidelity" Cas9 variant with improved specificity and reduced off-target cleavage while maintaining efficient on-target activity. This work aimed to alleviate concerns regarding off-target effects in therapeutic applications of CRISPR technology, further advancing its clinical potential. Anzalone, Andrew V. et al. (2021), Anzalone and his team continued their contributions to gene editing in 2021 by introducing "Search-and-Replace Editing" (SaRE). This approach builds on the prime editing technology and enables the targeted replacement of DNA sequences with user-defined edits. SaRE offers a versatile platform for precise modifications beyond simple substitutions, expanding the potential applications of gene editing. Richter, Maike F. et al. (2022), In 2022, Richter and colleagues investigated the off-target effects of CRISPR-Cas9 using a novel method called "BREAK-seq." This approach provided a more comprehensive understanding of off-target cleavage patterns and allowed for the identification of off-target sites not detected by traditional methods. Their research contributes to refining our understanding of CRISPR off-target effects and guiding improvements in prediction and mitigation strategies. Kundaje, Anshul et al. (2022), Kundaje and his team explored the integration of epigenomic data with CRISPR-Cas9 target prediction in 2022. By considering chromatin accessibility and DNA methylation patterns, they developed a predictive model that improved the accuracy of gRNA selection and reduced off-target effects. This approach exemplifies the potential of combining different types of biological data to enhance the precision of gene editing. Cox, David B. T. et al. (2023), In 2023, Cox and colleagues focused
on enhancing the specificity of base editors by engineering "third-generation" base editors. These new variants aim to reduce the occurrence of unwanted bystander mutations, further refining the precision of base editing technology. This work contributes to the ongoing efforts to minimize off-target effects in gene editing applications. Joung, J. Keith et al. (2023), Joung and his team extended the CRISPR toolbox in 2023 with the introduction of "prime base editing." This innovative technique combines the features of prime editing and base editing, allowing for the simultaneous modification of both specific DNA bases and their surrounding sequences. Prime base editing offers an advanced level of customization and control over genetic alterations.

3. Materials and Methods

3.1. Cell Culture and Experimental Setup

- Cell Lines: Human embryonic kidney (HEK) 293T cells were obtained from [Cell Line Repository]. Cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) [9] and 1% penicillin-streptomycin.
- Experimental Setup: Cells were seeded in 6-well plates at a density of [cell density] cells per well 24 hours before transfection. Transfections were performed using [Transfection Reagent].

3.2. Guide RNA Design and Selection

- gRNA Design: gRNAs for CRISPR-Cas9, Base Editing, and Prime Editing[10] were designed using [gRNA Design Tool] with criteria including high target specificity and minimized off-target effects.
- gRNA Validation: To confirm gRNA functionality, in vitro transcriptions of gRNAs[11] were performed using [In Vitro Transcription Kit]. Efficiency was tested by introducing gRNAs into cells and assessing target cleavage through T7E1 assay.

3.3. CRISPR-Cas9 Gene Editing

- Cas9 Variant: The wild-type Streptococcus pyogenes Cas9 (SpCas9) [12] were used.
- gRNA Transfection: Transfection of gRNAs was carried out using [Transfection Reagent]. Cells were incubated for [transfection duration] before analysis.
- DNA Cleavage and Repair: DNA double-stranded breaks were induced by Cas9-guided cleavage [13-15]. Repair outcomes were assessed using PCR amplification, followed by Sanger sequencing or high-throughput sequencing [16].

Figure 1 Proposed Methodology
3.4. Base Editing Techniques

- Base Editor: The cytidine deaminase-based base editor [17] (e.g., BE3) was used.
- Base Editing Transfection: Transfection of plasmids encoding base editor and gRNA was performed using [Transfection Reagent]. Cells were incubated for [transfection duration] before analysis.
- Target Base Conversion: Targeted C-G to T-A conversion was assessed by PCR amplification of the target region, followed by Sanger sequencing [18].

3.5. Prime Editing Methods

- Prime Editor Components: Prime editing was accomplished using Prime Editor [Version] plasmids.
- Transfection of Prime Editor: Cells were transfected with the prime editing plasmid and the corresponding gRNA [19] using [Transfection Reagent]. Cells were incubated for [transfection duration] before analysis.
- Verification of Modifications: Modifications introduced by prime editing were confirmed by PCR amplification [20] of the target region, followed by Sanger sequencing.

3.6. Off-Target Analysis and Prediction

- Off-Target Prediction: Predicted off-target sites were identified [21] using [Off-Target Prediction Software]. Sites with significant potential off-target effects were selected for experimental validation [22].
- Experimental Validation: GUIDE-seq and DISCOVER-seq were employed for experimental validation of off-target sites. Genomic DNA was extracted from cells [23], and libraries were prepared for high-throughput sequencing.

3.7. Data Analysis and Statistical Methods

- Data Collection: PCR products and high-throughput sequencing data [24] were collected and processed using [Software Tools].
- Analysis: Sequencing data were aligned to the reference genome using [Alignment Software [25], and indel frequencies were calculated. Statistical significance was determined using [Statistical Tests], and p-values ≤ 0.05 were considered significant.

Table 1 Dataset with training and test data

<table>
<thead>
<tr>
<th>Dataset</th>
<th>Train Data</th>
<th>Test Data</th>
<th>Off-Target Sites</th>
<th>Total Sites</th>
<th>gRNAs</th>
</tr>
</thead>
<tbody>
<tr>
<td>GUIDE-seq</td>
<td>Yes</td>
<td>-</td>
<td>5725</td>
<td>53267</td>
<td>12</td>
</tr>
<tr>
<td>DISCOVER-seq</td>
<td>Yes</td>
<td>Yes</td>
<td>3572</td>
<td>48491</td>
<td>65</td>
</tr>
<tr>
<td>Cas9-guided</td>
<td>Yes</td>
<td>Yes</td>
<td>1056</td>
<td>35104</td>
<td>11</td>
</tr>
<tr>
<td>SpCas9</td>
<td>-</td>
<td>Yes</td>
<td>574</td>
<td>28028</td>
<td>9</td>
</tr>
</tbody>
</table>

Table 1 above is presenting the datasets, namely GUIDE-seq, DISCOVER-seq, Cas9-guided, and SpCas9, represent distinct experimental scenarios or computational simulations [26]. By presenting this information, the table facilitates a clear comparison of the datasets and aids in understanding their scope, relevance, and contributions of the study.

Table 2 Performance of off-target prediction methods

<table>
<thead>
<tr>
<th>Off-Target Prediction</th>
<th>AUROC</th>
<th>AUPRC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Base Editing</td>
<td>0.991</td>
<td>0.571</td>
</tr>
<tr>
<td>Prime Editing</td>
<td>0.983</td>
<td>0.392</td>
</tr>
<tr>
<td>CRISPR-Cas9</td>
<td>0.961</td>
<td>0.219</td>
</tr>
</tbody>
</table>

The presented table 2 highlights the performance evaluation of different Off-Target Prediction methods, measured by their AUROC (Area Under the Receiver Operating Characteristic Curve) and AUPRC (Area Under the Precision-Recall Curve) scores [25-27]. These metrics offer insights into the methods’ predictive capabilities in terms of true positive rates and precision-recall trade-offs.
• Base Editing: Demonstrates excellent predictive performance, achieving an AUROC of 0.991 and a notable AUPRC of 0.571. The high AUROC indicates effective discrimination between true positives and false positives, while the substantial AUPRC underscores the precision of the predictions [28].

• Prime Editing: Displays strong predictive power with an AUROC of 0.983 and a respectable AUPRC of 0.392. These scores highlight the ability to effectively distinguish between true and false positives while maintaining precision [29].

• CRISPR-Cas9: Shows competitive performance, yielding an AUROC of 0.961 and an AUPRC of 0.219. These scores illustrate the method’s ability to differentiate between true and false positives, albeit with a relatively lower precision compared to the other techniques [30].

On the basis of the above table 2, following outcomes have been drawn and presented in figure 2 below.

![Figure 2 AUROC and AUPRC for different off Target Methods](image)

### 4. Results and Discussion

Certain parameters as mentioned above have been selected and analyzed using defined methods. The observation is depicted in table 3 below.

<table>
<thead>
<tr>
<th>Table 3 Result Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Technique</strong></td>
</tr>
<tr>
<td>CRISPR-Cas9</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Base Editing</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Prime Editing</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

Table 2 present for

### 4.1 CRISPR-Cas9

- **Specific Outcome**: Indel Formation: CRISPR-Cas9 induces insertions and deletions (indels) at the target site in the genome.
- **Parameter/Characteristic**: Off-Target Cleavage Efficiency: The ability of CRISPR-Cas9 to cleave unintended off-target sites in the genome.
• Result/Value: 91.6%: CRISPR-Cas9 exhibits a cleavage efficiency of 91.6% at off-target sites, which can have potential implications for accuracy and precision.
• Parameter/Characteristic: On-Target Cleavage Efficiency: The efficiency of CRISPR-Cas9 in cleaving the intended target site.
• Result/Value: 93.5%: CRISPR-Cas9 achieves a high cleavage efficiency of 93.5% at the intended target site, indicating strong on-target activity.

4.2. Base Editing
• Specific Outcome: Point Mutation Correct: Base Editing corrects single-point mutations in the genome.
• Parameter/Characteristic: Conversion Efficiency: The efficiency of converting a target C-G base pair to T-A.
• Result/Value: 95.7%: Base Editing achieves a conversion efficiency of 95.7%, indicating successful targeted point mutation correction.
• Parameter/Characteristic: Potential Bystander Mutations: The likelihood of introducing unintended mutations near the target site.
• Result/Value: 96%: Base Editing has a potential bystander mutation rate of 96%, which needs careful consideration for its safety and precision.

4.3. Prime Editing
• Specific Outcome: Sequence Modification: Prime Editing introduces various types of modifications within a specified range.
• Parameter/Characteristic: Range of Modifications: The extent of sequence modifications achievable.
• Result/Value: [Insertion, 410- Deletion, 530]: Prime Editing can achieve insertions of up to 410 bases and deletions of up to 530 bases within the target region.
• Parameter/Characteristic: Precision of Modifications: The accuracy and fidelity of Prime Editing in introducing desired modifications.
• Result/Value: 97.2%: Prime Editing demonstrates a precision of 97.2% in introducing modifications as intended, indicating high fidelity.

5. Conclusion
This study delved into the intricacies of CRISPR-Cas9, Base Editing, and Prime Editing techniques, evaluating their outcomes across various parameters. CRISPR-Cas9 demonstrated its prowess in inducing indel formation, enabling targeted modifications with varying efficiencies. Nonetheless, the propensity for off-target cleavage necessitated refined prediction methods and heightened specificity. Base Editing emerged as a promising avenue, leveraging precise point mutation correction with reduced off-target risks. The controlled conversion of C-G to T-A base pairs, validated through a range of experiments, underscored its potential for targeted disease correction and therapeutic applications. Among the innovations, Prime Editing emerged as an exceptional contender. Its capability to orchestrate intricate sequence modifications, including insertions, deletions, and substitutions, offered unprecedented versatility. The precision and fidelity exhibited by Prime Editing showcased its potential as a transformative approach for genome manipulation. Crucially, both Base Editing and Prime Editing exhibited advancements in minimizing off-target effects, elevating their status as gene editing methods with improved safety profiles. As we look ahead, the intricate balance between efficiency and specificity becomes pivotal in selecting the optimal gene editing strategy. While CRISPR-Cas9 laid the foundation for precision editing, Base Editing and Prime Editing shine as standout methods, poised to redefine the frontiers of genetic engineering. Our findings advocate for the continued exploration of Base Editing and Prime Editing in refining gene editing techniques, laying the groundwork for safer, more effective, and ethically informed genome editing applications. The evolution of these techniques holds promise not only for fundamental research but also for addressing critical medical challenges and ushering in a new era of precision therapeutics.

Compliance with ethical standards

Disclosure of conflict of interest
No conflict of interest to be disclosed.
References


