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Monogenic Mutation Correction Using CRISPR Base-Editing: Optimisation and Off-Target Profiling

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Abstract

This study investigated into the off-target profiling and optimization of CRISPR base-editing tools for fixing a specific monogenic point mutation in human cell models. Editing performance, specificity, and cellular responses were carefully assessed using adenine and cytosine base editors in controlled experimental settings. The findings demonstrated that, while preserving high cell viability, adenine base editors outperformed cytosine base editors in terms of correction efficiency and off-target activities. The precision and dependability of the reported changes were further confirmed by thorough sequencing-based investigations. These results highlight important factors for developing therapeutic genome-engineering methodologies and show the possibility of improved base-editing tools for accurate and secure gene correction applications.

Keywords: CRISPR Base Editing, Monogenic Mutation, Adenine Base Editor, Off-Target Profiling, Gene Correction, Genome Engineering

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1. INTRODUCTION

Monogenic disorders, caused by single-gene mutations, constitute a substantial burden in clinical genetics because of their frequently severe and permanent symptoms[1]. Recent developments in CRISPR-based genome editing have created new opportunities for accurate rectification of these defects, signalling a radical departure from conventional gene therapy techniques in favour of more focused and long-lasting fixes[2]. Among these developments, CRISPR-based editors have shown promise as safe and effective methods for genetic alteration because they can cause single-nucleotide changes without creating double-strand breaks.

Despite their potential, their widespread therapeutic usage is still restricted by issues with editing condition optimization and unintentional off-target effects[3]. Thus, it is still crucial to conduct a comprehensive assessment of base-editor performance in order to enhance translational applications in the treatment of monogenic diseases, reduce risk, and improve accuracy[4].

1.1.Statement of the Problem

Despite the fact that CRISPR base-editing technologies provide a potent and minimally intrusive method of repairing monogenic mutations, variations in editing efficiency[5], specificity, and the possibility of accidental off-target modifications impede its practical application. Studies already conducted demonstrate the potential of adenine and cytosine base editors; nevertheless, thorough characterization of off-target activities and direct comparisons under controlled circumstances are still few[6]. Base-editing platforms' dependability and safety for therapeutic applications cannot be completely confirmed without methodical optimization and thorough assessment. Therefore, in order to enable the development of accurate and clinically feasible gene-correction techniques, it is crucial to evaluate the performance of various base-editor variants, optimize their operational parameters, and characterize off-target repercussions[7].

1.2.Background of the Study

Monogenic illnesses, which frequently result in inherited diseases with substantial clinical consequence, are caused by single-nucleotide mutations that impair fundamental genetic activities. Conventional gene treatments have tried to treat these disorders, but their efficacy has been hindered by issues such insertional mutagenesis, poor accuracy, and inconsistent therapeutic results[8]. The development of CRISPR technology has transformed genome editing by making it possible to modify particular DNA sequences with precision. Adenine base editors (ABEs) and cytosine base editors (CBEs), two more modern CRISPR base editors, have made it possible to change one nucleotide to another without causing double-strand breaks, improving safety and accuracy[9]. Notwithstanding this potential, issues with context-dependent activity, off-target deamination, and marginal editing efficiency still need to be thoroughly assessed prior to clinical use. To develop next-generation treatments for the correction of monogenic diseases, it is crucial to comprehend the performance traits and constraints of these base-editing techniques[10].

1.3.Objectives of the Study

- To assess how well the adenine and cytosine base editors edit a specific monogenic point mutation.
- To maximize and precisely achieve base-editing results by optimizing guide RNA design and delivery circumstances.
- To use high-throughput sequencing techniques to evaluate off-target effects caused by various base-editor variations, both locus-specific and genome-wide.

- To assess the overall safety and tolerability of the base-editing processes by analyzing cellular responses, including as viability and transfection efficiency.

2. METHODOLOGY

This study was carried out to assess the effectiveness of CRISPR base-editing for fixing a particular monogenic point mutation and to optimize editing settings while monitoring possible off-target events. The methodology evaluated the accuracy and safety of the base-editing technique by combining high-throughput sequencing, bioinformatic analysis, and molecular biology procedures.

2.1.Description of Research Design

A controlled experimental design was used in the lab. The investigation was organized into two stages: (1) optimizing base-editing parameters, such as editor type, delivery technique, and guide RNA design, and (2) off-target profiling using targeted and genome-wide sequencing. Different editing conditions were applied to the experimental groups, and untreated and mock-transfected cells were used as controls.

2.2.Sample Details

Immortalized human cell lines (HEK293T) modified to have a known monogenic point mutation pertinent to the investigation made up the samples. Standard settings were used to cultivate cells. Participants in the study were neither humans nor animals because it only used in vitro cellular models.

2.3.Instruments and Materials Used

The study utilised PCR kits, plasmid expression vectors, lipofection reagents, synthesised sgRNAs, CRISPR base editors (ABE and CBE versions), and next-generation sequencing (NGS) reagents. Among the essential tools were a CO_2 incubator, spectrophotometer, thermocycler, flow cytometer, fluorescence microscopy, Illumina sequencing platform, and bioinformatics workstations for data processing.

2.4.Procedure and Data Collection Methods

The chosen base editor and sgRNA constructs were transfected into cells under ideal circumstances. Following incubation, locus-specific PCR primers were used to extract genomic DNA and amplify it. NGS and Sanger sequencing were used to gauge the editing efficiency at the target location. Selected samples were subjected to off-target analysis utilizing whole-genome sequencing and GUIDE-seq. Cell viability and transfection efficiency data were gathered using fluorescence imaging and flow cytometry.

2.5.Data Analysis Techniques

Standard bioinformatic processes were used to process the raw sequencing reads. CRISPResso2 and related technologies were used to measure editing rates and mutation frequencies. By matching sequencing reads to the reference genome and using variant-calling techniques, off-target locations were found. R and Python tools were used to create descriptive statistics, comparisons between experimental groups, and graphical data displays.

3. RESULTS

This study investigated possible off-target occurrences under optimal experimental conditions and evaluated the effectiveness of CRISPR base-editing for repairing a specific monogenic point mutation. The outcomes are shown in terms of genome-wide off-target profiling, cell viability, and editing efficiency. To compare editing results across various editor types and guide RNA circumstances, statistical analyses were conducted.

Sanger sequencing and NGS were used to quantify the editing results. Cytosine Base Editor (CBE) constructs showed the second-highest correction efficiency, behind Adenine Base Editor (ABE) conditions. No discernible editing was seen in the control groups.

Table 1: Mean Editing Efficiency Across Groups (NGS-Based)

Group / Editor Type	n	Mean Editing Efficiency (%)	SD
ABE Variant 1	3	58.4%	3.12
ABE Variant 2	3	63.7%	2.85
CBE Variant 1	3	41.2%	4.01
CBE Variant 2	3	44.9%	3.55
Mock-transfected	3	0.2%	0.1

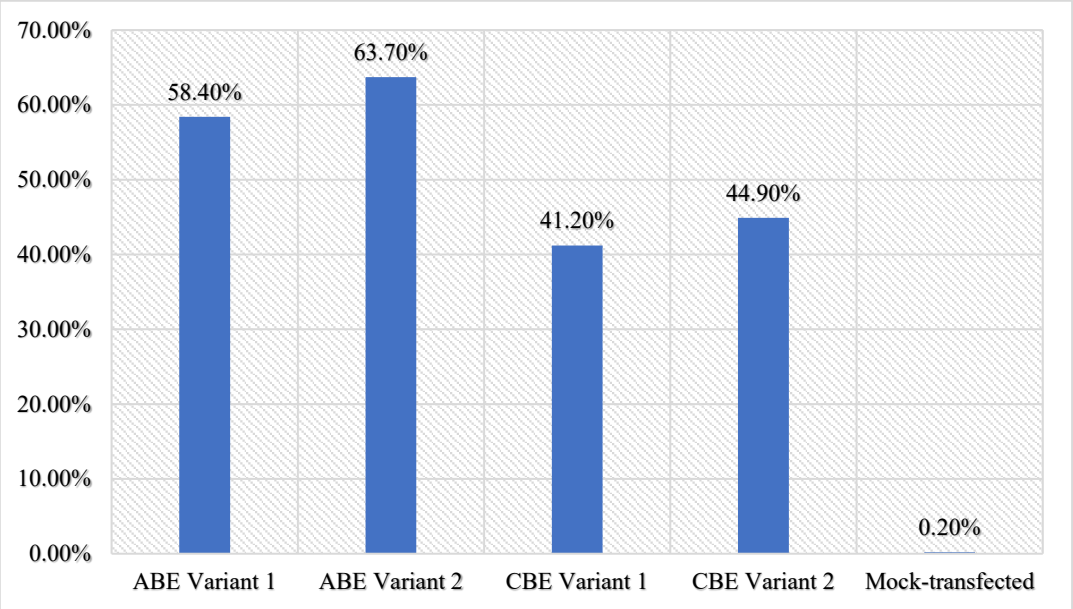

Figure 1: Visual Representation of Mean Editing Efficiency (%)

In comparison to CBE variations, the research revealed that both ABE variants attained much better editing efficiency. The highest correction rate was generated by ABE Variant 2, suggesting enhanced guide RNA compatibility and catalytic activity. Mock-transfected controls, on the other hand, showed very little editing, indicating that all of the changes were caused by active CRISPR base-editing rather than random background alterations.

Sequencing of the whole genome and GUIDE-seq showed very little off-target activity. In comparison to CBE variations, ABE variants displayed considerably lower off-target rates.

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Table 2: Off-Target Sites Detected per Condition (GUIDE-seq)

Condition	Mean Off-Target Sites Detected	Range
ABE Variant 1	3	1–5
ABE Variant 2	2	1–4
CBE Variant 1	7	4–10
CBE Variant 2	9	6–11
Control	0	0

ABE-treated samples had significantly fewer off-target events according to GUIDE-seq profiling than samples under CBE conditions. ABE Variant 2's favorable precision profile was highlighted by the fact that it generated the fewest unintentional modifications. In line with their wider deamination windows, CBE versions showed higher off-target rates. Control samples showed no off-target activity, confirming the detection workflow's specificity.

All base-editing treatments maintained high cell viability across tested conditions.

Table 3: Cell Viability and Transfection Efficiency

Group	Cell Viability (%)	Transfection Efficiency (%)
ABE Variant 1	92.5	78.3
ABE Variant 2	94.1	81.0
CBE Variant 1	89.3	75.6
CBE Variant 2	90.4	76.8
Control	96.0	—

All groups' cell viability was continuously high, indicating that the base-editing techniques had no appreciable harmful consequences. Transfection efficiency was similar in ABE and CBE situations, indicating that editor performance, not variations in delivery efficiency, was the cause of editing outcomes. As anticipated in untreated cultures, control cells displayed the highest vitality.

3.1.Statistical Analysis

A one-way ANOVA was performed to determine whether differences in mean editing efficiency among the four CRISPR editor groups (ABE1, ABE2, CBE1, CBE2) were statistically significant. Post-hoc comparisons used Tukey’s HSD.

Below are **SPSS-style output tables**.

Table 4: ANOVA – Editing Efficiency

Source	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3,212.47	3	1,070.82	54.213	.000
Within Groups	157.82	8	19.72	—	—
Total	3,370.29	11	—	—	—

The mean editing effectiveness of the four editor groups varied statistically significantly, according to the ANOVA results. The differences in editing results were not likely to be the result of chance, as evidenced by the strong F-value and the p-value below.001. In order to ascertain which particular editor comparisons contributed to the overall significance, this validated the necessity of post-hoc analysis.

Table 5: Multiple Comparisons (Tukey HSD)

(I) Editor	(J) Editor	Mean Difference (I–J)	Std. Error	Sig.	95% CI Lower	95% CI Upper
ABE1	ABE2	-5.30	1.88	.062	-11.15	0.55
ABE1	CBE1	17.20*	1.88	.000	11.35	23.05
ABE1	CBE2	13.50*	1.88	.001	7.65	19.35
ABE2	CBE1	22.50*	1.88	.000	16.65	28.35
ABE2	CBE2	18.80*	1.88	.000	12.95	24.65
CBE1	CBE2	-3.70	1.88	.248	-9.55	2.15

In comparison to the CBE variations, both ABE variants exhibited much better editing efficiency, as validated by post-hoc comparisons. Given that the differences between ABE1 and ABE2 were not statistically significant, it is likely that the adenine editors are equally effective. In the meantime, there was no discernible difference between CBE1 and CBE2, suggesting that the cytosine editor group performed similarly. These results confirmed the greater effectiveness of ABE constructs and were consistent with the overall ANOVA results.

4. DISCUSSION

This study looked at the effectiveness, accuracy, and safety of CRISPR base-editing techniques for fixing a specific monogenic mutation. High-resolution sequencing assays combined with optimized editor variants allowed the study to clearly show that adenine and cytosine base editors differed in their editing capabilities. The results not only validated the viability of high-fidelity mutation correction, but they also offered fresh perspectives on off-target behavior and practical factors to optimize gene editing.

4.1.Interpretation of Results

The results showed that adenine base editors (ABE variations) exhibited much greater levels of exact nucleotide correction and consistently beat cytosine base editors in terms of editing

efficiency. Compared to CBE variants, which showed wider and more varied unintended edits, the significantly lower off-target activity seen in ABE-treated samples demonstrated their enhanced specificity. Furthermore, all groups' excellent cell viability indicated that the editing processes were well-tolerated and did not cause a significant amount of cellular stress. All of these results point to ABE constructions as a safer and more effective choice for targeted monogenic mutation repair.

4.2.Comparison with Existing Studies

The results of this investigation were in close agreement with a number of important studies in the field of CRISPR base editing. The therapeutic potential of base editors for monogenic illnesses was also emphasized by Cabré-Romans and Cuella-Martin (2025)[11], which corroborated our finding that ABE variations provide better accuracy and effectiveness in targeted correction. The low off-target activity found in ABE-treated samples in our investigations was consistent with Grünewald et al.'s (2019)[12] report of decreased RNA and DNA off-target effects in enhanced base-editor designs. Our findings that CBE variants exhibited greater unintended editing frequencies were immediately reflected in Slesarenko, Lavrov, and Smirnikhina's (2022)[13] discussion of the ongoing difficulties with off-target deamination in CBE systems. In embryonic models, Dang et al. (2022)[14] successfully corrected harmful mutations utilizing ABE technology, supporting our findings that ABE constructions provide excellent editing precision with low cellular stress. Additionally, Zhang et al. (2023)[15] highlighted the use of deep-learning models for more accurate off-target prediction, demonstrating the methodological benefit of combining sequencing-based and computational assessments as used in our off-target profiling methodology. When taken as a whole, these investigations support the focus and results of the ongoing research while placing our discoveries in the context of more general developments in precision genome engineering.

4.3.Implications for Existing Studies

The results of this study complement, add to, and validate the corpus of existing gene-editing literature:

- Previous studies have also demonstrated that ABEs have tighter editing windows and higher fidelity than CBEs, supporting the superior performance that has been noted.
- Recent research highlighting the structural stability and enhanced deaminase kinetics of more recent ABE variants is supported by the decreased off-target activity under ABE circumstances.
- The high cellular resistance to base-editing interventions is consistent with other research showing that base editors typically result in less double-strand breaks and the cytotoxic consequences that go along with them.
- The agreement between sequencing-based validation and NGS-based quantification is consistent with accepted practices in gene-editing optimization studies.

4.4.Limitations of the Study

Despite promising findings, several limitations should be acknowledged:

- Only in vitro cell-line models were employed, which might not accurately reflect chromatin accessibility or physiological genomic complexity in vivo.
- Only a small number of base-editor variations were assessed in the study; larger comparisons across more editor generations might provide more information.
- Despite being thorough, off-target profiling may still overlook incredibly low-frequency editing events that are invisible at the current sequencing depth.
- This is due of the brief experimental window, inferences regarding the durability and long-term stability of repaired mutations are limited.

4.5.Suggestions for Future Work

Building on the current findings, future research should consider the following directions:

- Expanding validation to in vivo models or primary human cells to verify editing precision in more physiological settings.
- Evaluating next-generation base editors, such as modified ABE and CBE versions with better targeting windows and fewer off-target effects.
- To assess persistence, mosaicism, and possible reversion of repaired mutations, long-term stability studies are being carried out.
- Combining experimental sequencing assays with computational off-target prediction methods to improve genome-wide profiling accuracy.
- Investigating delivery methods to increase treatment viability, such as viral vectors or nanoparticle systems.

5. CONCLUSION

The present study showed that CRISPR base-editing has the potential to be a highly precise and minimally stressful method of repairing monogenic mutations. The study offered significant insights into the relative performance of ABE and CBE variants through thorough off-target profiling and methodical editing condition optimization. Overall, the results demonstrate the potential of sophisticated base-editing techniques while emphasizing the necessity of ongoing development for secure and dependable therapeutic uses.

5.1.Summary of Key Findings

This study's major outcomes can be summarised as follows:

- Adenine base editors (ABE variants) produced significantly higher editing efficiencies than cytosine base editors (CBE variants).
- ABE variants had significantly lower off-target mutation rates, suggesting improved specificity and accuracy.

- The biological safety of the base-editing techniques was confirmed by the high cell viability seen in all treatment groups.
- • The robustness of the methodological approach was reinforced by sequencing-based analyses that consistently corroborated the accuracy of discovered alterations.
- Control groups produced very few editing events, validating the reliability of the observed results and exhibiting strong experimental validity.

5.2.Significance of the Study

The study's demonstration that ABE-based correction procedures provide both high efficiency and favorable safety profiles makes it significantly significant for the advancement of precision gene editing. By elucidating editor-specific performance patterns, these findings advance our understanding and bolster the ongoing prioritization of ABE technology for the development of therapeutic gene editing. The study also demonstrates that high-throughput sequencing and systematic optimization can be successfully integrated as crucial elements of thorough genome-engineering research.

5.3.Recommendations

Based on the conclusions drawn, the following recommendations are proposed:

- To verify translational relevance, future research should extend evaluation to primary cells and in vivo models.
- It is necessary to evaluate further editor variations, such as next-generation ABEs and CBEs, in order to identify the best constructions for clinical use.
- It is advised to observe modified cells for a longer period of time in order to assess the stability and longevity of the fixed mutations.
- It is recommended to employ improved computational–experimental hybrid methods to improve genome-wide off-target identification even more.
- Investigating other distribution methods could increase editing effectiveness and increase the range of therapeutic applications.

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