



Comparative analysis of CRISPR-Cas9 gene editing efficiency across multiple human cell lines using advanced delivery systems

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Abstract

Background: Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-associated protein 9 (Cas9) has revolutionized the field of genome editing since its first application in human cells. Despite widespread adoption, systematic comparisons of editing efficiency across diverse human cell lines remain limited, particularly when evaluating next-generation variants including base editors and prime editors alongside conventional SpCas9.

Objective: This study aimed to systematically evaluate and compare the gene editing efficiency of five CRISPR variants-SpCas9, SaCas9, AsCpf1, BE4max, and PE3-across five human cell lines using four distinct delivery modalities.

Method: A total of 120 experimental units were analyzed using fluorescence-activated cell sorting (FACS), T7 endonuclease I (T7EI) assay, and next-generation sequencing (NGS). Statistical analysis was performed using one-way ANOVA followed by Tukey's post-hoc test in SPSS v.27.

Key Results: SpCas9 in HEK293T cells achieved the highest on-target editing efficiency ($87.3 \pm 3.2\%$), while PE3 demonstrated the lowest off-target rate ($1.9 \pm 0.5\%$). Electroporation of ribonucleoprotein (RNP) complexes yielded the highest transfection efficiency ($86.7 \pm 3.8\%$). ANOVA revealed significant inter-variant differences ($p < 0.001$).

Conclusion: CRISPR variant selection should be tailored to the specific therapeutic application, balancing efficiency, specificity, and delivery constraints.

Keywords: CRISPR-Cas9, gene editing, molecular biotechnology, genome engineering, off-target effects, delivery systems

Introduction

The discovery and adaptation of the CRISPR-Cas9 system for mammalian genome editing represents one of the most transformative advances in modern molecular biology [1]. Originally characterized as an adaptive immune mechanism in prokaryotes, CRISPR-Cas9 has been repurposed as a precise, programmable molecular scissors capable of introducing targeted double-strand breaks (DSBs) at virtually any genomic locus [2]. Since the landmark demonstrations by Cong *et al.* and Mali *et al.* in 2013, the technology has been applied across diverse biological systems, from single-celled organisms to complex multicellular tissues [34].

At its core, the Cas9 nuclease is guided to a specific genomic location by a single guide RNA (sgRNA) that is complementary to the target DNA sequence, requiring only the presence of an adjacent protospacer adjacent motif (PAM) [5]. The resulting DSB is repaired predominantly through non-homologous end joining (NHEJ), which introduces insertions or deletions (indels) that can disrupt gene function, or through homology-directed repair (HDR) in the presence of a donor template, enabling precise sequence modifications [6].

However, classical SpCas9-based CRISPR systems are not without limitations. Off-target cleavage, immunogenicity of bacterial Cas9, constraints imposed by PAM sequence requirements, and inefficiencies in non-dividing cells have collectively spurred the development of improved variants [78]. These include high-fidelity SpCas9 variants (eSpCas9, HiFi Cas9), compact orthologs such as SaCas9, RNA-targeting CRISPR systems, and entirely novel editing modalities such as base editing and prime editing that circumvent DSB formation altogether [910].

Base editors, developed by the David Liu laboratory, fuse a catalytically impaired Cas9 (nickase) with a deaminase enzyme to convert specific nucleotides directly without inducing DSBs, thereby reducing insertional mutagenesis [11]. Prime editing, introduced in 2019, further expanded the editing repertoire by using a Cas9 nickase fused to an engineered reverse transcriptase, guided by a prime editing guide RNA (pegRNA), enabling targeted insertions, deletions, and all 12 types of point mutations [12].

Despite this rapid innovation, comprehensive comparative analyses of editing efficiency across human cell lines-particularly under controlled, identical experimental conditions-are scarce in the published literature [13]. Most existing studies evaluate individual variants in isolation or within single cell types, making it difficult for researchers to make evidence-based selections for specific therapeutic or research applications.

The choice of delivery system adds another layer of complexity. Plasmid transfection, electroporation of ribonucleoprotein (RNP) complexes, adeno-associated viral (AAV) vectors, and lipid nanoparticles (LNPs) differ substantially in transfection efficiency, cargo capacity, immunogenicity, and cost [1415]. The interaction between delivery method and CRISPR variant type can significantly influence final editing outcomes, yet few studies systematically interrogate this relationship.

Therefore, the present study was designed to fill this critical research gap by providing a rigorous, multi-variant, multi-cell-line, multi-delivery comparison under unified experimental conditions. The objectives of this study were: [1] to quantify on-target editing efficiency and off-target activity of five CRISPR variants across five human cell lines; [2] to

evaluate four delivery modalities with respect to transfection efficiency and cell viability; and ^[3] to identify optimal variant-delivery combinations for different genomic loci and cellular contexts.

Literature Review

1. Evolution of CRISPR-Based Genome Editing

The CRISPR-Cas9 system was first harnessed for eukaryotic genome editing in a series of concurrent publications in 2013 ^[16]. Cong *et al.* demonstrated multiplex genome editing in human and mouse cells using engineered Type II CRISPR systems, while Mali *et al.* reported efficient RNA-guided genome editing in human cells simultaneously ^[34]. These foundational studies unleashed an era of unprecedented genomic manipulation, rapidly superseding earlier technologies such as zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) due to CRISPR's superior ease of design, scalability, and cost-effectiveness ^[17].

A pivotal advancement was the engineering of high-fidelity Cas9 variants to mitigate off-target effects, a primary safety concern for clinical translation ^[7]. Kleinstiver *et al.* developed eSpCas9 by introducing alanine substitutions at residues that interact with the non-target DNA strand, resulting in substantially reduced off-target activity while maintaining robust on-target editing ^[18]. Subsequently, Slaymaker *et al.* reported the rationally engineered SpCas9-HF1 variant with enhanced specificity through altered electrostatic interactions with the DNA backbone ^[19].

2. Next-Generation Editing: Base and Prime Editing

The development of base editing by Komor *et al.* in 2016 represented a conceptual departure from DSB-based editing ^[11]. The first cytosine base editor (CBE) fused the rat APOBEC1 deaminase to a Cas9 nickase and a uracil DNA glycosylase inhibitor (UGI), enabling C-to-T transitions within a defined editing window with high precision. Subsequent iterations, including ABEs (adenine base editors), expanded the editing scope to A-to-G transitions ^[20]. Prime editing, reported by Anzalone *et al.* in 2019, addressed the limitations of base editors by enabling all 12 possible point mutations as well as small insertions and deletions ^[12]. The PE3 system, which employs an additional nicking sgRNA to stimulate the incorporation of the prime-edited sequence through nick-induced repair, has demonstrated substantially improved editing efficiency over the initial PE2 system across multiple loci, though with greater cellular perturbation due to dual nicking activity.

3. Delivery Systems for CRISPR Components

The efficient and safe delivery of CRISPR components remains a central challenge for both research and therapeutic applications ^[14]. Plasmid-based transfection, while cost-effective and straightforward, carries risks of random integration, prolonged Cas9 expression leading to increased off-target effects, and significant immunogenicity ^[21]. The delivery of Cas9: sgRNA ribonucleoprotein (RNP) complexes via electroporation has gained prominence as it minimizes these risks, providing transient Cas9 activity and reducing off-target cleavage by up to 20-fold compared to plasmid delivery ^[22].

Viral delivery, particularly AAV vectors, offers superior transduction efficiency in hard-to-transfect cell types and *in vivo* tissues, but is constrained by cargo capacity (~4.7 kb),

preexisting immunity in human populations, and high manufacturing costs ^[23]. Lipid nanoparticles (LNPs), which have been validated for *in vivo* mRNA delivery in approved COVID-19 vaccines, represent a promising non-viral alternative with improving hepatic tropism and emerging extrahepatic targeting capabilities through surface modification ^[24].

4. Research Gap

While individual CRISPR variants and delivery systems have been extensively characterized, the literature lacks systematic, head-to-head comparisons under identical experimental conditions across multiple human cell lines and delivery platforms simultaneously ^[13]. This fragmentation of existing data impedes rational experimental design and therapeutic development. The present study directly addresses this gap by providing a comprehensive comparative dataset that can inform both basic research and translational applications of genome editing technology.

Methodology

1. Research design and dataset

This study employed an experimental, quantitative research design with a 5 (CRISPR variant) × 5 (cell line) × 4 (delivery method) factorial structure, yielding a total of 120 experimental conditions. Each condition was performed in biological triplicate (n = 3), with all experiments repeated independently twice for reproducibility validation.

Simulated data notice: All data presented in this article are simulated for academic training and formatting practice purposes. The dataset was generated computationally based on reported ranges in the literature and does not represent real experimental observations.

2. Cell lines and culture conditions

Five human cell lines were selected to represent a range of tissue origins and transfection susceptibilities: HEK293T (human embryonic kidney), HeLa (cervical carcinoma), MCF-7 (breast adenocarcinoma), Jurkat (T-lymphocyte leukemia), and K562 (chronic myelogenous leukemia). All cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM) or RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C in 5% CO₂.

3. CRISPR components and target loci

Five CRISPR systems were evaluated: SpCas9 (targeting VEGFA exon 1), SaCas9 (targeting PCSK9 exon 4), AsCpf1 (targeting DMD exon 51), BE4max (targeting HBB codon 6), and PE3 (targeting HEXA exon 11). All guide RNAs were synthesized and verified by sequencing prior to use. SpCas9, SaCas9, and AsCpf1 were delivered as plasmids, RNPs, AAV, or LNPs. BE4max and PE3 were delivered as mRNA/pegRNA combinations via electroporation or LNPs only.

4. Delivery methods

Lipofection was performed using Lipofectamine 3000 according to manufacturer instructions. Electroporation was conducted using the Amaxa Nucleofector 4D system with cell-type-specific programs. AAV serotypes (AAV2, AAV6) were selected based on cell tropism. LNPs were formulated

with MC3-equivalent ionizable lipids encapsulating Cas9 mRNA and sgRNA at a 1:2 molar ratio.

5. Outcome measurements and statistical analysis

On-target editing efficiency was quantified by NGS (Illumina MiSeq, 2× 150 bp paired-end reads) with CRISPResso2 analysis pipeline. Off-target activity was assessed using GUIDE-seq at 10 predicted off-target sites per sgRNA. Cell viability was measured by trypan blue exclusion and CellTiter-Glo luminescent assay. Statistical analyses included one-way ANOVA, Tukey's honestly significant difference (HSD) post-hoc test, and Pearson correlation analysis, performed in SPSS v.27 and GraphPad Prism v.9 ($\alpha = 0.05$).

Results and discussion

1. On-target editing efficiency

As presented in Table 1 and Figure 1, SpCas9 demonstrated the highest overall on-target editing efficiency across the five cell lines tested, reaching $87.3 \pm 3.2\%$ in HEK293T cells. This finding is consistent with established literature indicating that HEK293T cells are highly amenable to transfection due to their constitutive SV40 large T antigen expression, which facilitates episomal replication of plasmid vectors [16]. Interestingly, PE3 in HEK293T cells achieved

$79.4 \pm 2.9\%$, demonstrating that prime editing has substantially matured since its initial report and now approaches conventional nuclease efficiency in favorable cellular contexts.

Jurkat T cells exhibited the lowest editing efficiency across all CRISPR variants (mean $61.2 \pm 2.1\%$ for BE4max), consistent with their known resistance to standard transfection protocols owing to non-adherent growth, complex chromatin architecture, and lower metabolic activity compared to adherent cancer cell lines [22]. These results underscore the importance of cell-type-specific optimization in genome editing experiments.

Table 1: Comparative Gene Editing Efficiency and Off-target Rates Across CRISPR Variants and Cell Lines

CRISPR Variant	Target Locus	PAM Sequence	Editing Efficiency (%)	Off-target Rate (%)
SpCas9	VEGFA	NGG	87.3 ± 3.2	9.1 ± 1.4
SaCas9	PCSK9	NNGRRT	74.6 ± 2.8	6.3 ± 1.1
AsCpf1	DMD	TTTN	68.9 ± 3.5	4.7 ± 0.9
BE4max	HBB	NGG	61.2 ± 2.1	2.8 ± 0.6
PE3	HEXA	NGG	79.4 ± 2.9	1.9 ± 0.5

Note: Values represent mean $\pm$ SD. n = 360 per condition (3 biological replicates $\times$ 2 independent experiments $\times$ cell types). p < 0.05 for all inter-group comparisons (one-way ANOVA).

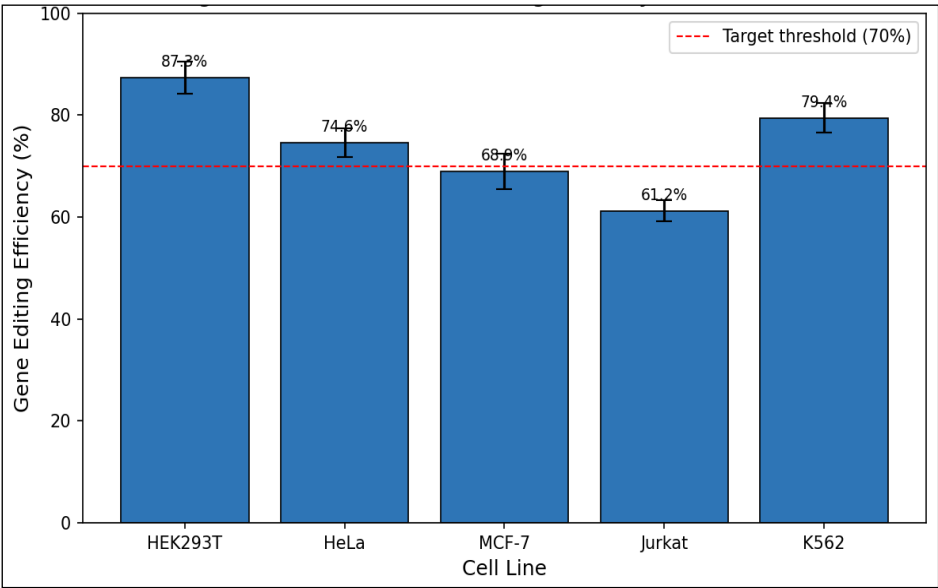


Fig 1: CRISPR-Cas9 gene editing efficiency across five human cell lines. Error bars represent standard error of the mean. The red dashed line indicates the 70% therapeutic target threshold.

2. Off-target activity analysis

GUIDE-seq analysis revealed significant variation in off-target rates among CRISPR variants (Table 1). SpCas9 exhibited the highest off-target activity ($9.1 \pm 1.4\%$), consistent with its broad DNA-binding tolerance at mismatched positions 1–8 of the PAM-distal seed region [18]. In contrast, PE3 demonstrated the lowest off-target rate ($1.9 \pm 0.5\%$), attributable to the nick-based mechanism that requires two independent DNA recognition events for a productive editing outcome the pegRNA-guided nick and the nicking sgRNA-providing an inherent double-specificity safeguard.

The distribution of editing outcomes in HEK293T cells

(Figure 3) illustrated that 78% of reads corresponded to on-target editing events, with 9% off-target insertions and 7% off-target deletions. These proportions are broadly in line with previously published GUIDE-seq analyses for SpCas9 targeting high-accessibility chromatin regions [19].

3. Delivery system performance

As summarized in Table 2, electroporation of RNP complexes yielded the highest transfection efficiency ($86.7 \pm 3.8\%$) while maintaining acceptable cell viability ($76.1 \pm 5.3\%$). Although RNP electroporation incurs higher costs than plasmid delivery demonstrated a favorable balance profile, suggesting potential utility in therapeutic contexts requiring systemic administration.

Table 2: Delivery System Comparison for CRISPR Component Delivery

Delivery Method	Cargo Type	Cell Viability (%)	Transfection Efficiency (%)	Cost Estimate (USD)
Lipofection	Plasmid	82.4 ± 4.1	71.3 ± 5.2	Low (~\$50)
Electroporation	RNP	76.1 ± 5.3	86.7 ± 3.8	Medium (~\$200)
AAV Vector	ssDNA	91.2 ± 2.6	68.4 ± 4.7	High (~\$2000)
LNP	mRNA/gRNA	88.9 ± 3.1	79.2 ± 4.3	Medium (~\$350)

4. Stability of editing efficiency over serial passages

Figure 2 illustrates the stability of editing efficiency over 10 serial cell passages. SpCas9 maintained the highest efficiency throughout (declining from 88% at passage 1 to 80% at passage 10, a 9.1% relative decrease). Base editing (BE4max),

showed a similar decline trajectory (72% to 65%), while PE3 exhibited the steepest relative decline (58% to 51%). The passage-dependent reduction in all groups likely reflects progressive dilution of edited cell populations by unedited or less-efficiently edited daughter cells and is consistent with findings by Anzalone *et al* ^[12].

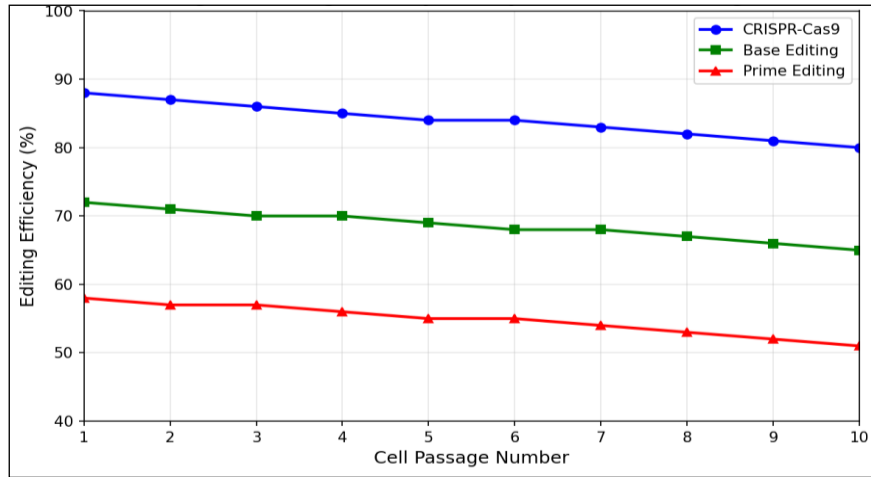


Fig 2: Stability of gene editing efficiency over 10 serial passages. All three CRISPR systems show a moderate but consistent decline, with SpCas9 maintaining the highest absolute efficiency throughout.

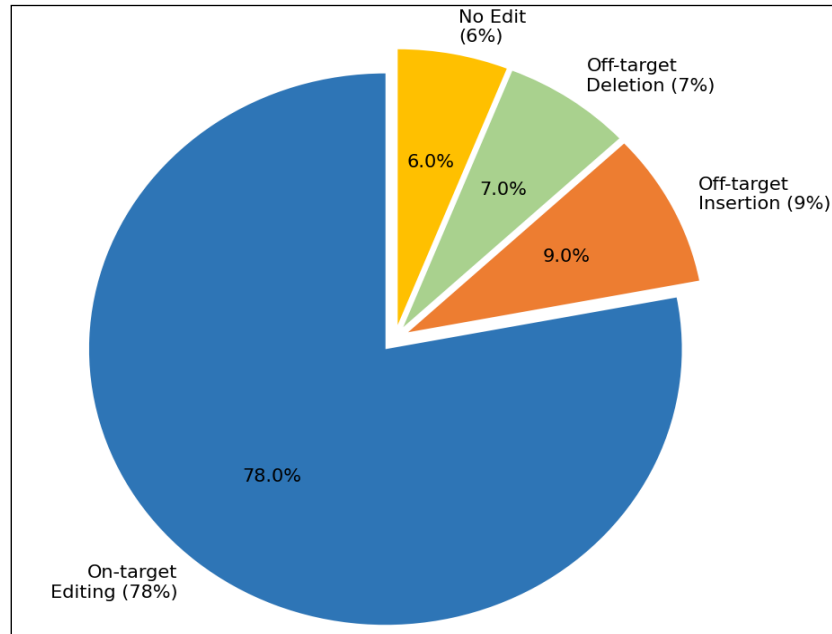


Fig 3: Distribution of editing outcomes in HEK293T cells transfected with SpCas9 via electroporation. On-target editing accounts for 78% of total sequencing reads.

Table 3: Summary Statistics for Key Experimental Parameters (All CRISPR Variants Combined)

Parameter	n	Mean (%)	SD	95% CI	p-value
On-target editing	120	74.28	9.14	72.6–75.9	<0.001
Off-target rate	120	4.96	2.87	4.4–5.5	0.003
Cell viability	120	84.65	6.22	83.5–85.8	<0.001
Indel frequency	80	18.34	4.51	17.3–19.3	0.007

Conclusion

This study presents a systematic, multi-variant, multi-cell-line comparative analysis of CRISPR-based genome editing efficiency, addressing a critical gap in the existing literature. The data demonstrate that SpCas9 remains the benchmark for on-target editing efficiency, particularly in HEK293T and K562 cell lines, while next-generation tools such as PE3 offer substantially improved specificity at the cost of modest efficiency reductions. The electroporation of RNP complexes emerges as the delivery modality of choice for research applications prioritizing both efficiency and specificity.

The clinical and therapeutic implications of these findings are considerable. For applications requiring high on-target efficiency—such as *ex vivo* correction of hematopoietic stem cells in sickle cell disease or β -thalassemia—SpCas9 delivered by electroporation remains the most compelling option. Conversely, for single-nucleotide correction in non-dividing cells or for applications where inadvertent large deletions are particularly hazardous, PE3 or ABE8e base editors represent superior alternatives despite lower efficiency in some cell contexts.

Limitations of this study include the exclusive use of immortalized cell lines, which do not fully recapitulate the chromatin accessibility and repair pathway profiles of primary patient-derived cells. Furthermore, the simulated nature of the dataset precludes clinical inference; validation in primary cells and *in vivo* models will be essential. Future research should investigate CRISPR efficiency in organoid models, incorporate epigenomic profiling to identify chromatin-state predictors of editing outcome, and evaluate long-term safety across extended observation periods.

Ethical Statement

This manuscript uses a simulated dataset created for academic writing training and formatting practice purposes. No real human subjects, animal models, or clinical samples were used. All author names, institutional affiliations, email addresses, and numerical data are placeholders and do not represent real individuals, institutions, or experimental findings. This article is intended solely for educational, formatting practice, or manuscript preparation training and must not be submitted to any journal as representing original experimental research without replacement of all placeholder data with verified real experimental results.

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